Inflammatory Mediators in the Lungs of Preterm Infants and their Role in the Development of Chronic Lung Disease

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Declaration

I hereby declare that this thesis has been my own composition. The experimental work was carried out predominantly by myself, as part of a research team and contributions from co-workers have been acknowledged, where appropriate, in the text. No part of this work has been submitted for any other degree.

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Finally thanks to my family and friends who are probably (but not quite) happier than me that it's finished.

Abstract

The progression to chronic lung disease in premature ventilated infants is now recognised as having several causes which together may lead to a poor respiratory outcome for the survivor. The premature lung, immature in structure and with a lack of surfactant and anti-oxidant enzymes, is poorly equipped to deal with the stress of ventilator barotrauma and oxygen toxicity. Recent studies have concluded that the pathology of chronic lung disease also involves a significant pulmonary inflammatory reaction and this is the subject of this thesis. Both clinical and laboratory studies were carried out with the emphasis on the early initiators and mediators of the inflammatory response.

The inflammatory response is orchestrated by the resident alveolar macrophages by production of cytokines in response to stimuli. These stimuli have not yet been fully identified although the genital mycoplasmas may have a role. The pro-inflammatory cytokines interleukin-1 (IL-1) and tumour necrosis factor-α (TNF-α), are early mediators and facilitate neutrophil migration by increasing the concentration of interleukin-8 (IL-8) from alveolar macrophages, and fibroblasts and epithelial cells lining the lung. IL-8 is one of the family of alpha-chemokines, and it acts as a chemoattractant to neutrophils circulating peripherally. TNF-α increases the permeability of the endothelial lining allowing the circulating neutrophils to migrate into the lung. The IL-8 then activates the neutrophils which degranulate releasing powerful enzymes and produce highly toxic oxygen radicals, both of which exacerbate the tissue damage initiated by the ventilation.

The clinical pilot study enrolled 26 infants and measured IL-8, TNF-α and inflammatory cells in bronchoalveolar lavage samples. The results demonstrated that it may be possible to predict a group of babies at extremely high risk for developing chronic lung disease by early measurement of IL-8 in tracheal secretions.

To further investigate the role of the genital mycoplasmas, *U. urealyticum and M. hominis,* a randomised study using an effective antibiotic, erythromycin, was carried out. A low grade untreated infection could persistently stimulate an *in vivo* inflammatory response, but we also wished to investigate the reported anti-inflammatory properties of erythromycin. The inflammatory mediators and cells present in bronchoalveolar lavage were measured. The infection rate in the study was lower than expected (12% compared to 30% in a pilot study) and did not correlate with outcome. There was also no correlation between outcome and treatment and the inflammatory response, as measured by IL-8, did not correlate with outcome as it had in our pilot study.

Surfactant has been an elective rescue treatment for infants with respiratory distress syndrome, but there is debate as to whether natural or synthetic surfactants are more appropriate. As part of a randomised trial of a natural and synthetic surfactant taking place on our unit, bronchoalveolar lavage samples both pre and post surfactant were analysed for cytokines and cells. Our results showed that Curosurf produced a significantly lower inflammatory response compared to Exosurf 24 hours after administration but there was no correlation between surfactant type and development of chronic lung disease.

The cell population present in the lung effluent has been an area of controversy. Conventionally it is thought that neutrophils are the main cells implicated in lung damage, but longer term fibrosis would be under macrophage control. Most studies employ a simple differential stain to evaluate cell types, but a recent study showed that the use of this stain was inappropriate in sections of lung post-mortem tissue as it underestimated the number of macrophages present. Standard cytospin smears of bronchoalveolar lavage samples were compared using a differential stain and an immunocytochemical stain which relies on monoclonal antibodies to identify specific cell surface markers and is less subjective. Our results confirm that the differential stain identifies neutrophils but it significantly under-estimates cells of the monocyte/macrophage lineage.

Previous *in vitro* studies have demonstrated the ability of the genital mycoplasmas to stimulate lung cells to produce cytokines. Laboratory investigations were carried out to further elucidate and quantify the response of A549 human lung epithelial cells to *Ureaplasma urealyticum*, and an other genital isolates, a Gram-negative (*Eschericia coli*). The cells were stimulated in the presence of TNF-α and high oxygen concentrations, and with mediators used in the clinical management of these infants, including surfactant, antibiotics and steroids.

Summary

The progression to chronic lung disease in premature ventilated infants is now recognised as a multi-factorial disease with recent studies investigating pulmonary inflammatory reactions. Both clinical and laboratory studies were carried out for this thesis with the emphasis on the early initiators and mediators of the inflammatory response. These include interleukin-1 (IL-1) and tumour necrosis factor-α (TNF-α), which facilitate neutrophil migration by increasing the concentration of interleukin-8 (IL-8) released from alveolar macrophages, fibroblasts and epithelial cells. IL-8 activates neutrophils which contribute to the tissue damage initiated by ventilation.

The clinical pilot study enrolled 26 infants and measured IL-8, TNF-α and inflammatory cells in bronchoalveolar lavage samples. The results demonstrated that it may be possible to predict a group of babies at extremely high risk for developing chronic lung disease by early measurement of IL-8 in tracheal secretions.

To genital mycoplasmas, *U. urealyticum and M. hominis,* may have a role in the initiation of the inflammatory response. To further investigate their role, a randomised study using erythromycin was carried out. Not only is erythromycin an effective antibiotic, but has also been reported as an anti-inflammatory in adult lung disease. The inflammatory mediators and cells present in bronchoalveolar lavage were measured. The infection rate in the study was lower than expected (12% compared to 30% in a pilot study) and did not correlate with outcome. There was also no correlation between outcome and treatment and the inflammatory response, as measured by IL-8, did not correlate with outcome as it had in our pilot study.

Surfactant has been an elective rescue treatment for infants with respiratory distress syndrome, but there is debate as to whether natural or synthetic surfactants are more appropriate. As part of a randomised trial of a natural and synthetic surfactant taking place on our unit, bronchoalveolar lavage samples both pre and post surfactant were analysed for cytokines and cells. Our results showed that Curosurf produced a

significantly lower inflammatory response compared to Exosurf 24 hours after administration but there was no correlation between surfactant type and development of chronic lung disease.

The cell population present in the lung effluent has been an area of controversy. Most studies employ a simple differential stain to evaluate cell types, but it has recently been shown that the use of this stain was inappropriate as it underestimated the number of macrophages present. Standard cytospin smears of bronchoalveolar lavage samples were compared using a differential stain and an immunocytochemical stain which is less subjective. Our results confirm that the differential stain identifies neutrophils but it significantly under-estimates cells of the monocyte/macrophage lineage.

Previous *in vitro* studies have demonstrated the ability of the genital mycoplasmas to stimulate lung cells to produce cytokines. Laboratory investigations were carried out to further elucidate and quantify the response of A549 human lung epithelial cells to *Ureaplasma urealyticum*, and an other genital isolate, a Gram-negative (*Eschericia coli*). The cells were stimulated in the presence of TNF-α and high oxygen concentrations, and with mediators used in the clinical management of these infants, including surfactant, antibiotics and steroids.

Abbreviations

ANOVA	analysis of variance test
AM	alveolar macrophage
AsM	assay medium
APAAP	Alkaline phosphatase anti-alkaline phosphatase
BAL	bronchoalveolar lavage
B0	total binding expressed as a percent of the total counts
BPD	bronchopulmonary dysplasia
BSA	Bovine Serum Albumin
CI	confidence intervals
CLD	chronic lung disease
cpm	counts per minute
DMEM	Dulbecco's minimal essential medium
EDTA	ethylenediaminetetraacetic acid
ELF	epithelial lining fluid
ELISA	enzyme linked immunosorbent assay
FBS	foetal bovine serum
FM	freezing medium
GM	growth medium
ICAM	intercellular adhesion molecule
IL	interleukin
IL-1β	interleukin-1β
IL-8	Interleukin-8
MCP	macrophage chemotactic protein
MIP	macrophage inhibitory protein
NIBSC	National Institute for Biological Standards and Controls
NICU	Neonatal Intensive Care Unit
NSB	non-specific binding
PMN	polymorphonuclear
PBS	phosphate buffered saline
RDS	respiratory distress syndrome

ROS	reactive oxygen species
SAPU	Scottish Antibody Production Unit
SE-BTS	South East Blood Transfusion Service, Edinburgh
Sem	standard error of the mean
Stdev	standard deviation
TBS	Tris buffered saline
TNF-α	tumour necrosis factor-α
UAL	Ureaplasma urealyticum broth

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1. Introduction

1.1 Normal Lung Development

At birth, the human infants' lungs must efficiently mediate gas exchange between blood and air. To do this they have to have developed *in utero* to expand sufficiently on inhalation without rupturing, neutralise toxic oxygen radicals and allow gas exchange across a respiratory membrane that had recently held fluid. Lung development can be separated into two distinct but related processes, growth and maturation, where growth refers to the physical size, weight and structural developments and maturation refers to the ability of the lung to function (DiFiore *et al.*,1994).

1.1.1 Structural Development

Human lung development starts in the fourth week after fertilisation and ends in early childhood with the mature organs. There are five pre-natal stages from conception to birth (DiFiore *et al.*,1994) though the divisions are not sharp, rather they blend into one another and there is variation between individuals. The *embryonal* stage begins as a diverticulum from the foregut which later cleaves distally to form the lung buds. The buds continue to elongate and at 6 weeks form the bronchopulmonary segments lined with an epithelium. It is the interactions of the epithelium with the mesenchyme which continues to promote the branching of the lung. This concludes at 8 weeks.

The *pseudoglandular* stage lasts from the 7th to 16th week. The bronchioles continue to divide and by the 16th week the complete bronchial tree has formed. Any further increase in bronchial size is due to elongation not branching.

The *canalicular* phase extends from the 17th to 26th weeks and results in the formation of the gas exchanging capability of the lung. The blind ended, epithelial lined airways continue to extend into the mesenchyme where the capillaries form a network around the distal air passages. The alveolar epithelium begins to differentiate; the cytoplasm thins, the air-blood barrier narrows and the gas exchanging cells, the type I pneumocytes appear at 20-22 weeks. The surfactant producing cells -the type II

pneumocytes - appear just after this differentiating from cells in the epithelium (Chinoy *et al.*,1994).

During the *saccular* phase from the 26th to 36th weeks there is thinning of the airway walls and a decrease in the interstitial tissue as the peripheral airways continue to expand. The interstitium with its capillary network is thus sandwiched between adjacent airways which have saccules attached to the end. These saccules grow and divide and eventually become the alveolar ducts and sacs late in the saccular phase. Surfactant secretion from the type II cells also begins during this stage.

The final *in utero* stage is the *alveolar* starting at 37 weeks. This lasts well into the post-natal period. Early studies suggested that no alveoli were present initially, but recently it is recognised that alveoli do form *in utero* although ~ 80% of alveoli formation occurs postnatally. So even at birth the lungs have a substantial amount of structural development to undergo until they are fully mature.

1.1.2 Lung Maturation

Two major biochemical events occur within the lung which are essential for efficient function, surfactant and anti-oxidant enzyme production. As both these events happen at roughly the same time (approximately 30 to 32 weeks gestation) it has been speculated that they are under the same corticosteroidal control (Frank *et al.*,1985).

Surfactant

By 28 weeks gestation, the alveolar epithelium has differentiated into Type I and Type II pneumocytes and steroids from the adrenal stimulate the lung fibroblasts to produce an oligopeptide known as fibroblast-pneumocyte factor (Scott and Das., 1993, Post *et al.*, 1984) which stimulates surfactant synthesis from Type II cells.

Surfactant is a complex lipopotein, the major component of which is dipalmitoylphospatidylcholine (DPPC). There are four proteins A, B, C and D the structures of which have been recently reviewed (Creuwels *et al.*,1997). Together these reduce the surface tension at the alveolar air/liquid interface in the peripheral lung and contribute to the alveolar stability associated with normal respiration preventing the collapse of the alveoli. Surfactant lipids suppress the immunological

activities of alveolar macrophages (AM) (Shimizu *et al.*,1988) and may also have an immunomodulatory role.

The four proteins can be separated into two groups; two hydrophilic and two hydrophobic. The two hydrophobic proteins SP-B and SP-C maintain the stabilisation of the surface film by promoting rapid insertion of phospholipids into the monolayer (Baatz *et al.*,1990), and the correct ordering of the lipids in the monolayers (Perez-Gil *et al.*,1992) respectively.

The two hydrophilic proteins SP-A and SP-D have important functions in addition to reducing surface tension. SP-A, the most abundant protein in surfactant, has a role in regulating the uptake and secretion of surfactant (Ryan *et al.*,1989), opsonisation of inhaled particles (Malhotra *et al.*,1993) and bacteria (Kalina *et al.*,1995) to facilitate alveolar macrophage phagocytosis, and stimulating proinflammatory cytokine production (Eizioni.,1994). SP-D, the most recently identified protein, has a similar structure to SP-A and its functions also seems related to lung defence. It can opsonise Gram-negative bacteria (Lim *et al.*,1994) and induce production of oxygen radicals from alveolar macrophages (van Iwaarden *et al.*,1992) and has been suggested to scavenge LPS which may be important in prevention of septic shock (Creuwels *et al.*,1997).

Anti-Oxidant Enzymes

Exposure to increased oxygen concentrations at birth enhances the cellular production of reactive oxygen species (ROS) such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH^-) all of which can cause direct tissue damage. To combat this the lungs have antioxidant enzymes; superoxide dismutase (SOD), catalase and glutathione peroxidase which scavenge or detoxify these ROS. In animal models these enzymes appear late in the third trimester of gestation and their concentration increases rapidly over a very short time (Frank *et al.*,1987; review). In humans this pattern does seem to be repeated with SOD (Dobashi *et al.*,1993). At 36 weeks gestation there is antileucoprotease present in the bronchial epithelium (Willems *et al.*,1988) which is a potent inhibitor of elastase enzymes.

1.1.3 Cellular Structure of the Bronchioles and Alveoli

The cellular structure of the bronchioles is shown in Fig 1.

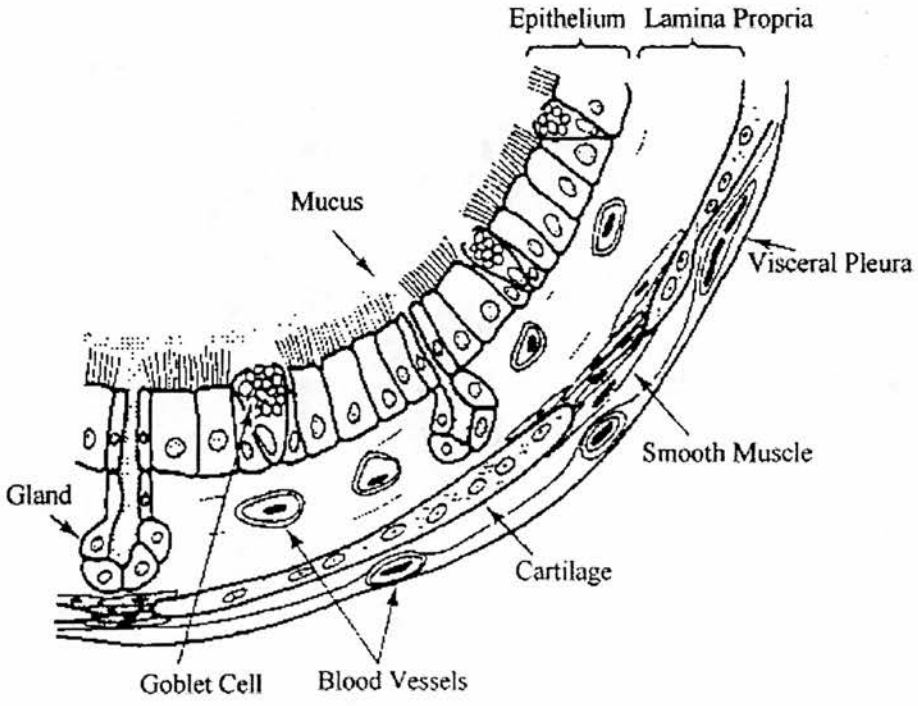


Figure 1-1: Diagram of the bronchial structure showing the ciliated epithelial cells which facilitate the movement of the mucus which is secreted by the goblet cells. Taken from (Widdicombe and Davies., 1991).

The walls of the alveolar ducts consist of a thin spiral of collagen and elastin and are likened to a coil of a spring: they lengthen on inspiration and close on expiration. The epithelium that lines the air side of the bronchiole is covered in mucus which is secreted by specialised cells (Jeffrey *et al.*,1992; review). Goblet cells and the less frequently found epithelial "serous" cell both secrete mucus. They are also responsible in adults for repair of the injured respiratory mucosa as they can divide into ciliated cells and other epithelial cells (Jeffrey *et al.*,1992). The mucus is distributed up the epithelium by the many columnar ciliated cells which beat in a regulated manner to

move the mucus and any debris it has caught from inspiration up to larynx where it is expectorated or swallowed. The mucus also serves other purposes in that it warms inhaled air, keeps the lining moist and is an effective anti-bacterial. The bronchioles end in the alveoli which are the gas exchanging part of the lung (see Fig 1.2).

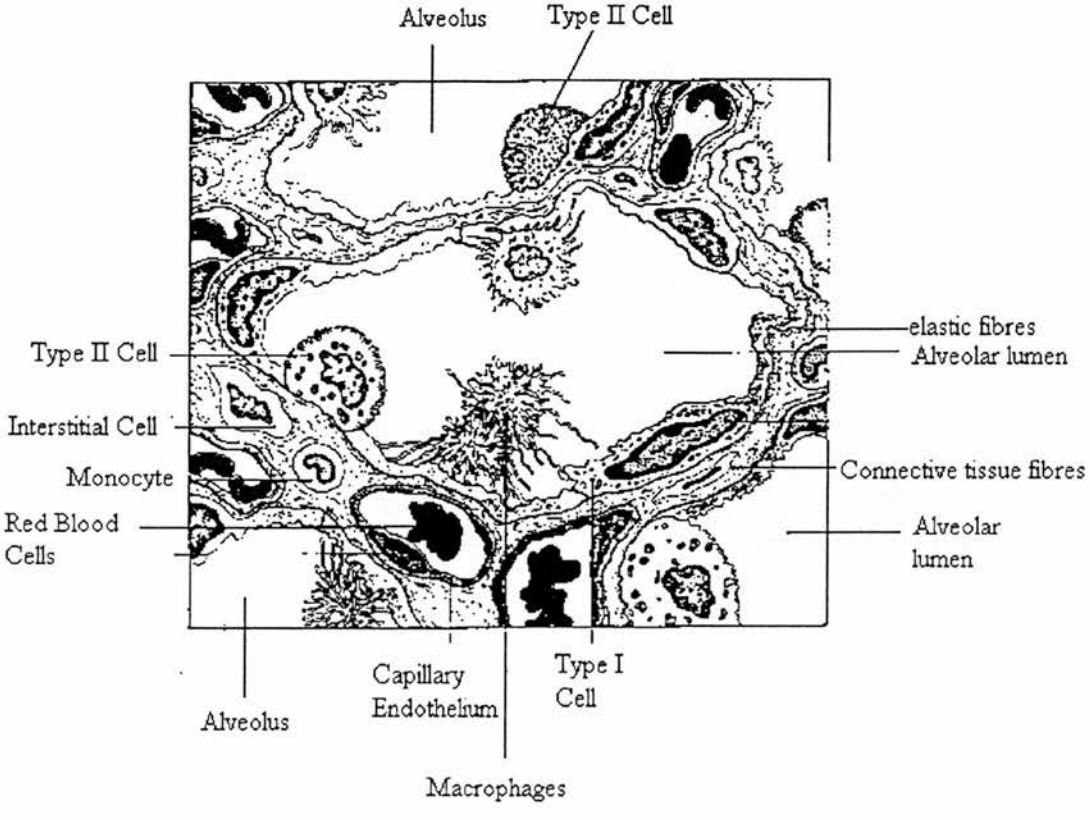


Figure 1-2: Schematic drawing of a section of lung tissue showing an alveolus and surrounding capillaries, as well as various cell types. Taken from (van Wynsberghe, Noback, and Carola.,1995).

The alveoli are box-like structures with one open side, and their overall structure is designed for maximum gas exchange. Surface tension must be reduced for adequate distensibility and this is achieved by pulmonary surfactant which is secreted by Type II cells. There is a single complete epithelium extending throughout all the alveoli which is continuous with that of the airways. It is separated from the underlying connective tissue by a basement membrane. The alveolar wall is rich in blood capillaries and on

one side the capillary is closely applied to alveolar epithelium. Here the endothelial and epithelial membranes lie very close and the air/blood barrier is at its thinnest approximately 0.15μm. Type I and II cells are both present in the alveoli. Type I cells have few cytoplasmic granules and have cytoplasm capable of spreading long distances from the nucleus. Some may penetrate the wall of the alveoli through the Kohn pores and so contribute to the lining of more than one alveolus. Their function is to provide a complete but thin covering preventing fluid loss and facilitating gas exchange but they are easily damaged. Type II cells cover much less of the surface than Type I and usually occupy the "corners" of the alveolus. They have numerous microvilli, secrete surfactant and can differentiate into type I cells should the need arise.

1.1.4 Immune Cells

The alveolar macrophages are the most abundant immune cells in the lung constituting 80 - 90% of the total immune cell population. The remaining 10% is made up of mostly lymphocytes with a very few polymorphonuclear cells. AM are present at the air-tissue interface in the alveoli and alveolar ducts where they scavenge debris and spent surfactant. They are in a unique position in that they are in constant contact with airborne antigens and must therefore exert control on their responses to them. In this respect they are similar to other macrophages at a mucosal surface such as the gut and share many similarities in phenotype with them.

They are a heterogenous population within the normal lung (Spiteri and Poulter.,1991; Rich *et al.*,1987) with cell surface markers identifying at least 3 distinct types of cells (Spiteri and Poulter.,1991); an inducer phenotype $RFD1^{+}/RFD7^{-}$, a suppressive phenotype $RFD1^{+}/RFD7^{+}$, and a classic macrophage phenotype $RFD1^{-}/RFD7^{+}$. The suppressor phenotype is important in normal lung functioning and prevents the dendritic cells from antigen-presentation (Pollard and Lipscomb.,1990; Holt *et al.*, 1993) and the T-cells from proliferating (McCombs *et al.*,1982; Bilyk and Holt.,1995), though not from becoming activated (Upham *et al.*,1997). Their role in scavenging spent surfactant may also promote their immunosuppressive properties (Speer *et al.*,1991; Thomassen *et al.*,1994; Zimmerman.,1995).

Blood monocytes replace the resident AM population and do not have a suppressive $RFD7^+$ phenotype (Poulter *et al.*,1986) and have been shown in gut inflammation to be capable of secreting many cytokines including IL-1β and TNF-α (Rugtveit.,1997). The heterogeneous population of the normal lung may reflect the acquisition of the suppressive phenotype as the monocytes mature *in situ*.

1.2 Inflammatory Responses Within the Lung

When an inflammatory response is required the AM must co-ordinate and control the response which may require the recruitment of specialist cells for efficient clearing of the organisms. They must also ensure that the response lasts no longer than necessary; shutting down the response, clearing away debris and promoting healing

1.2.1 Alveolar Macrophages

During a local inflammatory response, activated monocytes infiltrate from the blood and secrete cytokines. This influx of non-suppressive monocytes allows a T-cell mediated response by increasing the number of antigen presenting cells in the lungs. The cytokines granulocyte monocyte colony stimulating factor and TNF-α secreted by these monocytes may alter the responsiveness of the resident AM (Bilyk and Holt, 1993; Bilyk and Holt, 1995), allowing them to overcome their suppressive phenotype, and participate in the inflammatory process.

Also released are the pro-inflammatory cytokines IL-1β and TNF-α which have pleiotropic effects, promoting the up-regulation of adhesion molecules on the pulmonary endothelium (Falconer *et al.*,1995b; Chinoy *et al.*,1994b) and causing the release of chemokines. Both of these events trigger the infiltration of circulating neutrophils into the lung, which is detailed in the following sections.

1.2.2 Adhesion Molecules

The endothelium expresses adhesion molecules in response to cytokine signals and these regulate the influx of immune cells into the lung. They are a highly sophisticated set of cell surface molecules (See Table 1-1 below) which are specific to cell types

without which the lung would be unable to recruit cells capable of dealing with pathogenic organisms.

Table 1-1: Leucocyte-Endothelial Cell Adhesion Molecules (Taken from Brown, 1997)

Leukocyte receptor	Leukocyte expression	Endothelial counter-receptor	Function
Selectins			
L-selectin	All	Glycam-1, PSGL-1, others	Rolling
Integrins			
αMβ2 (Mac-1, Mo-l, CDllb/CD18)	Monocytes	ICAM-1 (CD54) ICAM-2 (CD102)[a] fibrinogen	Firm adhesion Transmigration
αLβ2 (CD11a/CD18)	All	ICAM-1 ICAM-2	Firm adhesion Transmigration
α4β1	All [b]	VCAM-1 (CD106)	Rolling and firm adhesion
α4β7	Mucosal Lymphocyte subset	MadCAM	Rolling and firm adhesion
αvβ3	All	PECAM (CD31)	Transmigration
Ig Superfamily			
IAP (CD47)	All	Thrombospondin	Transmigration
PECAM	All	PECAM, αvβ3	Transmigration
Others			
PSGL-1	PMN, monocytes, some lymphs	P-selectin (CD62P) L-selectin (CD62l) E-selectin (CD62E) E-selectin	Rolling
ESL	PMN	E-selectin	Rolling
CD44	All	Hyaluronate	?

[a] The role of MAC-1 interaction with ICAM-2 in transendothelial migration is unknown.
[b] While α4β1 has an important role in transendothelial migration of monocytes and lymphocytes, its role in PMN transendothelial migration is uncertain.
Abbreviations: NK, natural killer cells; PMN, polymorphonuclear neutrophil.

There is an ordered sequence of events which determine the flow of cells into the lung and they begin with the selectins, which allow neutrophils circulating in the blood to attach and "roll" along the endothelium. There are three known selectins; P, E and L-

selectin which mediate rolling, but each also has a distinct role which probably contributes to the finer points of cellular recruitment. L-selectin is expressed constitutively by leucocytes and P-selectin is stored in compartments within endothelial cells and rapidly translocated to the cell surface upon activation (Wagner.,1993). Both these selectins can therefore respond almost immediately to changes in endothelial cell surface receptors. E-selectin expression on the endothelium happens only after transcription and translation and does not appear until hours after P-selectin. In mediating neutrophil migration into the lung P- and L-selectin would therefore appear to be required early (Mayadas *et al.*,1993), whereas E-selectin is involved in later events.

Once the selectins have appeared on the endothelial surface neutrophils attach but the speed of the blood flow causes intense shear stresses. The selectin-ligand bonds form rapidly but are also easily broken and this has the effect of the neutrophil "rolling" along the endothelium, being taken in the direction of the blood flow. This attachment to the endothelium is not stable until another group of surface adhesion molecules becomes involved; the integrins.

The integrins are a family of transmembrane cell surface proteins that bind to the cytoskeleton and communicate extracellular signals. Their bond with the leucocyte ligands are much stronger than that of the selectins but they cannot form under the shear stress conditions the blood flow creates. Therefore the binding of the leucocytes via the selectins is a necessary prerequisite to integrin binding.

The endothelial cells up-regulate expression of ICAM-1 in response to the pro-inflammatory cytokines (Bloeman *et al.*,1993; Rothlein *et al.*,1988), which bind to the leucocytes via their LFA-1 or Mac-1 integrins. Once the leucocytes are held firmly to the endothelium the begin to migrate into the interstitium of the lung, against a gradient of chemokine, which guides them to the site of inflammation. The AM release other factors such as leukotriene B_4 which increase microvascular permeability (Ford-Hutchinson *et al.*,1984) and assist the transendothelial migration of leucocytes.

Thus endothelial responses to cytokines will upregulate microvascular adhesion mechanisms.

1.2.3 Chemokines

Chemokines are a family of basic heparin-binding proteins 7 - 10Kd in size that share significant sequence identity at the amino acid level. They are released in response to the pro-inflammatory cytokines and cause the activation and chemotaxis of specific cell types.

Based on conserved sequences of 4 cysteines at their NH_2 terminal end, several sub-groups can be defined. The two major groups are the CXC or α-chemokines, which have two cysteines separated by any other amino acid residue and the CC or β-chemokines which have two adjacent cysteines. These sub-groups are also specific for cell types. The α-chemokines are specific for neutrophils and include IL-8. The β-chemokines are specific for mononuclear cells and include MIP-1α and β, MCP-1, 2 and 3 and RANTES. Their specificity allows the recruitment of different types of effector cells at the site of an inflammation. The other sub-groups are the C family, of which lymphotactin is the only member at present, and CX_3C (Bazan *et al.*,1997) which comprises only neurotactin.

The family of α-chemokines is diverse and can be separated into two major sub-groups: the ELR CXC chemokines and the non-ELR CXC chemokines (Rollins.,1997). The ELR is a motif formed by three amino acids (glutamate-leucine-arginine) between the N-terminus and the first cysteine. The ELR CXC chemokines have predominantly neutrophils as their target cells and include IL-8, the GRO family, epithelial cell-derived neutrophil-activating peptide (ENA-78) and the platelet basic proteins which have varying degrees of potency *in vitro*.

IL-8 was the first of the α-chemokines to be described and has three forms with identical biologic activity (Baggiolini *et al.*, 1989). It is biologically active as a dimer and a monomer (Rajarathnam *et al.*,1994) and acts via a seven-transmembrane-domain (7TM) receptor, of which there are two expressed by neutrophils (CXCR1 and CXCR2). CXCR2 has a high affinity for all CXC chemokines but CXCR1 has a

high affinity for IL-8 only (Baggiolini *et al.*,1994). IL-8 binding to either receptor triggers Ca^{2+} changes and chemotaxis, but phospholipase D activation and respiratory burst are only triggered by CXCR1 (Jones *et al.*,1996). Thus IL-8 can affect cells differently depending on what receptors they are expressing.

The β-chemokines are likewise a large family of related chemokines whose primary target cell is monocytes, although most of its member affect T lymphocytes and eosinophils (Rollins.,1997). Monocyte chemoattractant protein (MCP-1) is the most potent chemokine of those currently studied (Uguccioni *et al.*,1995) .

In lung inflammation IL-8 is arguably the most important chemokine, being produced in greater quantities and faster than MCP-1 from monocytes (Liebler *et al.*,1994), although animal studies have shown that MIP-1α is important as an autocrine stimulator of AM and maintains high levels of TNF-α (Ward.,1996).

PMN entering the lung microvascular bed are retained exclusively within the capillaries (Lien *et al.*,1987) where they can respond rapidly to signals from the AM. Once the neutrophils have entered the alveolar space and are activated they phagocytose particulate matter or produce lytic enzymes and reactive oxygen species to kill invading pathogens.

1.2.4 Extracellular Matrix

The extracellular matrix (ECM) surrounds and supports the bronchial tree and is composed of collagen, fibronectin, elastin, proteoglycans and laminins (Comper & Laurent.,1978). It is essential in regulating the normal lung and its interaction with matrix enzymes allows necessary developmental stages as well as normal growth (Woessner.,1991, Matrisian.,1990). It interacts with pulmonary cells and growth factors and cytokines, many of which are produced during an inflammatory response.
The proteoglycans are composed of a protein core with many glycosaminoglycan (GAG) chains attached (Kjellen & Lindahl.,1991). They have highly diverse functions ranging from mechanical support as well as interacting with processes such as cell

adhesion. Most of these functions are dependent on proteins binding to the GAG chains. Hyaluronic acid (HA) is one of many of the GAG family, but is particularly important in the wound healing. During development the lung concentration of hyaluronan decreases with a concomitant increase in macrophage intracellular levels of hyaluronan (Underhill et al 1993). In fetal rabbits (Mast et al., 1992) high concentrations of HA decreased fibroblast infiltration and capillary formation but showed an organised collagen deposition. In adults HA levels are low and it has been speculated that HA may down-regulate the supply of growth factors such as TGF-β and PDGF thus preventing the wound healing process as seen in adults.

IL-1 and TNF-α promote the production of the metalloproteinases (MMPs) (Arend and Dayer.,1998) which results in degradation of the ECM and the basement membrane. During acute RDS associated with an inflammatory response, GAGs are degraded (Murch.,et al 1996a) and this is likely to increase pulmonary leakage and permeability as well as decrease compliance. The relationship between ECM and the pulmonary cells is also disrupted and the effects can be devastating in the restructuring of the lung. It is vital therefore that there is regulation of an ongoing inflammatory response, and that the response lasts only as long as it is required to remove the threat.

1.2.5 Down-Regulation of the Inflammatory Response

Once the danger has been eliminated a down-regulation of the inflammatory response is essential to prevent continued recruitment of activated immune cells and damage and degradation of the ECM. It is achieved in many ways. The alveolar macrophages actively suppress the response by producing interleukin-10 (IL-10) (Thomassen *et al.*,1996) in response to TNF-α (Wanidworanum *et al.*,1993) which inhibits further TNF-α release, down-regulates ICAM-1 expression on epithelial cells (Mulligan *et al.*,1993) and directly inhibits further IL-8 release from neutrophils (Cassatella *et al.*,1993). The AM are also stimulated to produce transforming growth factor-β (TGF-β) which prevents neutrophil migration by down-regulating the expression of

E-selectin on the endothelial cell surface (Gamble *et al.*,1998) and directly inhibits the production of IL-8 from endothelial cells (Smith *et al.*,1996).

The effector cells may become desensitised to cytokines by a reduction of surface receptors (Colditz and Movat.,1984) and IL-8 is thought to become inactive by binding to cells (Kunkel *et al.*,1991). The neutrophils are only short lived effector cells and therefore die at the site of the inflammation. To prevent them leaking their highly active cell contents into the lung and promoting further damage they undergo apoptosis (Savill *et al.*,1989). This is a controlled suicide in which their cell surfaces display proteins recognised by AM which then phagocytose and dispose of the debris of these cells without provoking further inflammation.

1.2.6 Repair of Damage

After an inflammatory insult damaged cells must be replaced and damaged tissue must be repaired. The fibroblasts proliferate stimulated directly by the macrophage derived cytokines platelet derived growth factor (PDGF) and transforming growth factor-β (TGF-β) (Thornton *et al.*,1990; Kovacs.,1991; Kovacs and DiPietro.,1994). These growth factors also increase synthesis of collagen and fibronectin (Matrisian.,1990; Ignotz *et al.*,1987) to replace the degraded matrix components, and increase cell affinity for collagen and fibronectin (Matrisian.,1990). They achieve this by upregulation of integrins (Ignotz and Massague.,1987; Ignotz *et al.*,1989) which are involved in cell-matrix and cell-cell interactions. These interactions are important in the remodelling process following injury.

To prevent further matrix degradation fibroblasts and AM are induced to produce tissue inhibitors of metalloproteinases (TIMPs) (Edwards *et al.*,1987).

The whole inflammatory response is normally held in check by a series of stringent controls designed to remove harmful stimuli without causing damage to the host. However it is clear that many pulmonary diseases are caused by an imbalance in one or more of the factors that regulate the inflammatory response, and this is discussed further in the following chapters.

1.3 Premature Birth

Premature birth results in an immature lung structure with a thick respiratory membrane which is however more permeable to small molecules. There are less terminal airspaces and a greatly reduced surface area for gaseous exchange (Langston *et al.*,1984). The chest wall is highly compliant and does not allow the generation of sufficient pressure for efficient lung inflation and lung fluid levels are higher than at term birth. In infants born ≤ 30 weeks gestation, surfactant (Avery & Mead.,1959) and antioxidant enzyme (Grigg *et al.*,1993) production are deficient, and these infants are born with respiratory distress which may require ventilation and a high oxygen supplement.

1.3.1 Ventilation

Mechanical ventilation may be vital for infant survival, but the high inspired oxygen required and the barotrauma ventilation produces are both likely to cause damage to an immature lung. The increased oxygen concentrations increases the cellular production of reactive oxygen species (ROS) which can cause direct tissue damage if not effectively removed. Antioxidant enzymes which do this job do not develop until after about 30 weeks gestation and so the preterm infant is deficient in the defence mechanisms designed to prevent oxidative damage.

The barotrauma of forced ventilation facilitates oxygen delivery, but with each breath, collapsed alveoli are ripped open and there may be disruption of the bronchiolar and alveolar epithelium. The immature cell lining is more permeable to small molecules and this is greatly increased by mechanical ventilation.

1.3.2 Surfactant Deficiency and Respiratory Distress Syndrome

The main role of surfactant is to reduce surface tension at the air/alveolar interface and to prevent the collapse of the alveoli. Once ventilated, infants may require ventilation for only a few days after which time their oxygen requirement declines and they recover with no long term effects. There is a group of infants however whose

RDS is complicated by other factors such as pneumothorax or pulmonary haemorrhage. These infants remain ventilated for prolonged periods of time and develop chronic lung disease of prematurity or bronchopulmonary dysplasia.

1.4 Chronic Lung Disease of Prematurity

Chronic lung disease or bronchopulmonary dysplasia has been defined in two ways;

i) a baby who has been ventilated and has an oxygen requirement at 28 days with abnormalities on x-ray

ii) a baby who has been ventilated and has an oxygen requirement at 36 weeks corrected gestational age with abnormalities on x-ray

Only the second definition predicts abnormal findings at follow-up in infants (≤ 30 weeks gestation (Shennan *et al.*,1988).

In this thesis chronic lung disease of prematurity or bronchopulmonary dysplasia will be referred to as chronic lung disease (CLD). For the definition of chronic lung disease used for clinical studies presented here, please refer to the individual chapters.

Classically chronic lung disease has been seen as the disordered repair of damaged tissues, the damage having been caused by treatment for RDS. The principle factors associated with this disease are:

i) prematurity

ii) respiratory failure

iii) oxygen toxicity

iv) barotrauma

It has recently been recognised, however, that together these factors stimulate an inflammatory response within the lungs which starts within hours of birth (references below) and which has even been speculated to start *in utero* (Ghezzi *et al.*,1997). Infection with the genital mycoplasmas has also been suggested as a possible chronic stimulant of the inflammatory response.

In infants that develop chronic lung disease the inflammatory response is persistent, resulting in long term hospitalisation and frequent respiratory problems in infancy. This inflammatory response and the role of the genital mycoplasmas is explored more fully in the following sections.

1.5 Preterm Neonatal Immune Responses

The immune system has two distinct defences; innate and adaptive. The innate response is the first line of defence against foreign antigens, whereas the adaptive system is antibody mediated and involves specific recognition and memory by cells. Newborn infants, term and preterm are more susceptible to infection and this may be related amongst other things to a deficiency in the neutrophil storage pool in the bone marrow (Liau *et al.*,1996) and that these neutrophils are functionally immature (Silver *et al.*,1996).

1.5.1 Functions of Neonatal Phagocytes

In healthy term infants cord blood monocytes are much slower at phagocytosis than adult cells (Schuit *et al.*,1980) and alveolar macrophages undergo significant biochemical, morphologic and functional postnatal development (Bellanti *et al.*,1979), with their ability to kill phagocytosed microbes only developing during the first postnatal month (Bellanti *et al.*,1979). Cord blood monocytes also produce significantly less of the pro-inflammatory cytokines IL-8 (Rowen *et al.*,1995) and TNF-α (Gerdes *et al.*,1992), whilst the data on IL-1β is not as clear they have been reported as producing less IL-1β (Gerdes *et al.*,1992) or similar levels to adults (Pillay *et al.*,1994). They also have significantly reduced cell surface TNF-α receptors (Chheda *et al.*,1996) and so are unable to respond as well as mature cells. They produce less leukotriene B_4 (Rowen *et al.*,1995) and IL-10 (Cheda *et al.*,1996) and do not respond as well as adults to IFN-γ (Marodi *et al.*,1994).

Preterm infants monocytes isolated from cord blood produce significantly lower TNFα (Weatherstone *et al.*,1989; Gerdes *et al.*,1992), IL-6 (Yachie *et al.*,1992) and IL-8 (Schibler *et al.*,1993) to various stimuli than term or adult monocytes. IL-1β has

been shown to be reduced (Gerdes *et al.*,1992) or equal (Weatherstone *et al.*,1989) to term/adult levels, but there is no correlation between gestational age, birthweight and TNF-α/IL-1 levels (Gerdes *et al.*,1992).

TNF-α is in part responsible for the fever seen in inflammation so this lower level of TNF-α may be why preterm infants don't get fever. However, it is not just poor TNF-α production which fails to stimulate IL-8 production, as exogenous TNF-α stimulated preterm cord monocytes show decreased IL-8 (Schibler *et al.*,1993) and IL-10 (Chheda *et al.*,1996) production.

Normal PMN are important effector cells but neonatal PMN have an inability to mobilise free Ca^{2+} ions intracellularly (Santoro *et al.*,1995) and this is an important trigger of chemotactic and phagocytic functions in PMN. Accordingly, studies on chemotaxis and phagocytosis have shown these cells are deficient (Falconer *et al.*,1995b; Hill.,1987; Eisenfeld *et al.*,1994; Santos *et al.*,1993).

The motility of preterm PMN cord blood cells is more severely impaired than term infants (Eisenfeld *et al.*,1994). L-selectin is normally associated with adhesive functions of neutrophils and is expressed constitutively. Leucocytes from preterm infants have decreased resting levels of L-selectin compared to adults, but there is no inability to up-regulate expression on stimulation (Rebuck *et al.*,1995). Mac-1, which mediates firm binding to the endothelium, is seriously deficient on the surface of preterm neonates neutrophils and monocytes (McEvoy *et al.*,1996) and is poorly up-regulated upon stimulation compared to adults (Torok *et al.*,1993).

The FcRIII is a member of the immunoglobulin superfamily and is important in binding opsonised bacteria and triggering the oxidative burst. In a normal fetus FcRIII is at low levels until 33 weeks gestation when it rapidly rises to reach adult levels at birth. Neonates have significantly less FcRIII on their neutrophil cell surface compared to adults (Falconer *et al.*,1995a) but whether this is due to shedding of FcRIII more easily or being unable to transport FcRIII from vesicles within the cell where it is stored (Payne *et al.*,1993a) is unclear. A recent study (Carr *et al.*,1992a) measured soluble FcRIII in fetal and preterm plasma and found that preterms have low levels at birth but within the first 3 weeks of life have achieved adult levels. This

suggests a programmed increase in protein synthesis not a sudden increase in receptor release and this is probably in response to external stimuli. PMN random migration in preterm infants has reached the level of term infants by 3 weeks postnatal age, but chemotaxis is still reduced in preterm infants (Usmani *et al.*,1991; Carr *et al.*,1992). It seems likely that birth causes maturation of PMN functions, with chronological age being more important to PMN functionality than gestational age.

Preterm PMN respiratory burst activity, important in generating reactive oxygen species, is severely impaired compared to term infants (Jaswon *et al.*,1994) but study populations are very heterogeneous with some neonatal PMN able to produce high concentrations of H_2O_2 (Ueda *et al.*,1992).

1.6 Studies on Lung Inflammatory Mediators in Premature Neonates

Recent studies have focused on the measurement of inflammatory mediators in the bronchoalveolar lavage fluid of neonates ≤ 30 weeks gestation in an attempt to correlate levels with outcome. Bronchoalveolar lavage is a routine procedure in intubated infants involving the squirting of sterile saline down an endotracheal tube with the subsequent removal of the saline and lung fluid by suction. It is performed routinely, and requires no ethical permission unless the procedure differs from the routine, and the sample collected can be analysed for cells and mediators (see "Chapter2 p30"). The measurement of cells is generally done by differential stain, whilst the fluid can be measured for levels of inflammatory mediators.

1.6.1 Cytokine Responses

Previous studies have not used exactly the same protocol for bronchoalveolar lavage, nor have they followed exactly the same analysis of samples. This makes comparisons between studies difficult. However, within 24 hours of birth there is a clear rise in markers of inflammation such as IL-1β (Jones *et al.*,1996; Liau *et al.*,1996), IL-6 (Bagchi *et al.*,1994), IL-8 (Jones *et al.*,1996), MIP-1α (Murch *et al.*,1996b) and fibronectin (Watts *et al.*,1992b) in infants with RDS compared to those without. IL-

10 is undetectable within the first 96 hours of life in preterm infants, but present in term infants (Jones *et al.*,1996).

At 2-3 weeks infants that later develop chronic lung disease have elevated soluble ICAM-1 (Kotecha *et al.*,1995; Silver *et al.*,1996), TNF-α (Murch *et al.*,1992; Bagchi *et al.*,1994), IL-8 (Groneck *et al.*,1994a; Kotecha *et al.*,1995a), C5a (Groneck *et al.*,1994a), fibronectin (Watts *et al.*,1992b), IL-6 (Ng.,1993), IL-1β (Murch *et al.*,1996b) and MIP-1α (Murch *et al.*,1996b). There are no control groups in these studies as infants that recover are not ventilated and this makes drawing conclusions difficult. However, the persistence of these mediators suggests an ongoing inflammatory response which is doubtless contributing to tissue damage and fibrosis.

1.6.2 Cellular Response

The cellular influx is equally rapid with neutrophils predominating, though there is debate as to whether macrophages, which are essential for the progression to fibrosis, are also present early on.

Merrit *et al* (1981) described the cellular influx in bronchoalveolar lavage in infants that had RDS and subsequent chronic lung disease and divided them into three classes. Infants that had no lung disease or RDS at birth generally had no immune type cells but between 3 to 10 days there was a rapid PMN influx. After ~ day 8 macrophages were the predominant reactive cell types. In infants with RDS that developed into chronic lung disease PMN numbers remained high, although macrophages were present, and there was microscopic evidence that these cells were reactive; they had abundant mitochondria in their cytoplasm. Subsequent studies using differential stains for cell identification have confirmed that a rapid neutrophil influx occurs within 48 hours, which in resolving RDS are replaced by macrophages but which persist in infants developing chronic lung disease (Ogden *et al.*,1984; Arnon *et al.*,1993).

Murch *et al* (1996b) do not share this view. They point out that neutrophils are easier to identify on differential stain compared to the heterogeneous monocyte/macrophage population. They investigated monocytes/macrophages using cell surface markers and concluded that in the group developing chronic lung disease, that

monocytes/macrophage numbers are significantly elevated as early as 48 hours postnatal age and remain so throughout the course of their stay. This was in contrast to the neutrophils which were only evident at day 3 onwards, but were also significantly raised in the chronic lung disease group. Macrophages and their products are important in the development of fibrosis (Finkelstein *et al.*,1992; Sanders *et al.*,1994; review) and given the disordered repair of tissues seen in chronic lung disease it is not surprising that they will be evident early on.

Once an inflammatory response is no longer required, neutrophil apoptosis is an important method of clearing activated cells to prevent them for doing damage to surrounding tissue. Grigg *et al* (1991) reported neutrophil apoptosis in 8 newborn infants after 3-5 days ventilation. This small study, however, does not correlate this to the clinical outcome of these infants and further studies will be required to establish whether this process is defective in infants developing chronic lung disease.

1.6.3 Neutrophil Elastase

The presence of such a large number of active neutrophils in the lung results in significant production of powerful enzymes such as elastase which attack the extracellular matrix. Elastase has also been shown to degrade surfactant protein A (SP-A) (Liau *et al.*,1996) which reduces surfactants ability to adsorb and increases surface tension. In infants with RDS that develop chronic lung disease elastase activity is significantly raised compared to infants with resolving RDS (Merrit *et al.*,1983). Moreover, high elastase levels in preterm lungs are not associated with α-proteinase inhibitor activity (Speer *et al.*,1993) the main deactivator of elastase. This elastase/proteinase inhibitor imbalance is likely to result in chronic lung inflammation and tissue damage (Ohlsson *et al.*,1992; Ogden *et al.*,1984).

1.7 Potential Infectious Initiators of the Inflammatory Response

1.7.1 Genital Mycoplasmas

The mycoplasmas are the smallest free-living bacteria. They have no rigid cell wall but their cytoplasmic contents are enclosed by a well developed plasma membrane. This makes them resistant to antibiotics such as penicillin that interfere with the integrity of the cell wall. The mycoplasmas are surface parasites of mucosal membranes; the respiratory and genital tracts. They attach via surface adhesins (Henrich *et al.*,1993) and their infectivity is greatly reduced when monoclonal antibodies to these adhesins are applied (Smith *et al.*,1994). Once attached they provoke an inflammatory response and are thought to damage host membranes by the production of hydrogen peroxide, though this is still speculative. They are Gram negative but stain poorly, and are not detected using the routine bacteriological media employed in our unit.

The genital mycoplasmas comprise *M. hominis* and *U. urealyticum*. *M. hominis* is roughly 100 - 300 μm whereas *U. urealyticum* is only 15 - 30μm. Both these organisms are commonly found in the vagina of sexually active asymptomatic women and are probably transmitted during sexual intercourse. *U. urealyticum* has been isolated from the genital tract of ~ 70% of pregnant women, and the rate of vertical transmission to preterm infants has been reported as up to 55% (Sanchez.,1993). Transmission is not affected by the mode of delivery, but is significantly increased if chorioamnionitis is present.

1.7.2 Genital Mycoplasmas and the Development of Chronic Lung Disease

In recent years there has been increasing evidence for a role of the genital mycoplasmas in prematurity and disease of newborns (Cassell *et al.*,1993). Whilst these organisms have been associated with histological chorioamnionitis, they have not been associated with premature labour or birth (Eschenbach.,1993). In four studies reviewed by Wang *et al* 1993, infection with the genital mycoplasmas was not shown to be causal of chronic lung disease, but they were isolated more often from the respiratory tract of infants who developed chronic lung disease compared to those who did not. Differences in study design and taking into account other aetiological factors did not affect the relative risk in the infected group of developing chronic lung

disease. Infection rates in these studies varied between 17 - 30% for *U. urealyticum* isolated from bronchoalveolar lavage samples (Payne *et al.*,1991; Wang *et al.*,1988; Cassell *et al.*,1988). All of these studies showed an association between *U. urealyticum* colonisation and the development of chronic lung disease, with infants < 1250g birthweight at an increased risk. In contrast, Heggie *et al* 1994 reported an infection rate of the genital mycoplasmas in bronchoalveolar lavage of 17%, but in infants between 26 and 29 weeks gestation there was no correlation with chronic lung disease (defined at either 28 days or 36 weeks corrected gestational age).

A pilot study carried out within our unit (Iles *et al.*,1996b) found 30% of a group of preterm infants < 30 weeks had tracheal aspirates positive for *M. hominis* and/or *U. urealyticum,* and of these infants 87% developed chronic lung disease compared to 41% of non-infected infants ($p = 0.019$).

Whilst the evidence for an association between the genital mycoplasmas and chronic lung disease is strong, the mechanism of the association is still speculative. We hypothesised that if these organisms are causal, the problem of chronic lung disease might be significantly reduced by antibiotics effective against these organisms.

1.7.3 Genital Mycoplasmas and the Inflammatory Response

The absence of a cell wall on the genital mycoplasmas means that they have no lipopolysaccharide and the major antigenic determinants appear to be cell membrane glycolipids and proteins. An antigen capable of great variation has been isolated on *U. urealyticum* which is predominantly recognised by humans during infection (Zheng *et al.*,1994).

Only a few clinical and *in vitro* studies have shown their ability to provoke an inflammatory reaction. Infants colonised with *U. urealyticum* show increased IL-1β, IL-8 and neutrophil counts in bronchoalveolar lavage samples (Groneck *et al.*,1996). In a study of 99 infants, those colonised with the genital mycoplasmas had higher levels of inflammatory mediators (including IL-8 and IL-6) on days 1 and 5 of life than non-colonised infants (Groneck *et al.*,1994b). Colonisation with *U. urealyticum* has also been shown to increase PMN counts in tracheal aspirates (Payne *et al.*,1993b) and white blood cell counts (Ohlsson *et al.*,1993).

In vitro studies have shown *U. urealyticum* can provoke IL-6 and IL-8 release from fibroblasts (Stancombe *et al.*,1993). Mycoplasma species have been shown to induce TNF-α from a monocytic line (Sugama *et al.*,1990), IL-6 from human monocytes (Quentmeier *et al.*,1990) and a range of cytokines from immunocompetent cells (Stuart.,1993).

1.8 Chronic Inflammation

Whilst neonatal phagocytes appear to have impaired chemotaxis *in vitro*, clinical studies show that a large influx of phagocytes does occur in ventilated preterm infants lungs. On stimulation however neonates may be responding with functionally immature phagocytes which may be unable to effectively remove a bacterial infection in the lungs due to impaired phagocytosis and microbial killing (Bellanti *et al.*,1979). Tissue damage caused by barotrauma, hyperoxia and surfactant deficiency will contribute to the problem by stimulating more leucocytes to migrate into the lungs and these cells will continue to produce TNF-α, IL-1β, IL-8 and MIP-1α. The neutrophils and AM will produce toxic oxygen radicals which cannot be neutralised, and the balance of MMPs and TIMPs may be altered (Ricou *et al.*,1996) affecting the stability of the extracellular matrix. Unable to down-regulate the response through cytokines such as IL-10 (Cheda *et al.*,1996), surfactant or antioxidant enzymes, preterm infants will then suffer from a chronic inflammatory response. This will promote the proliferation of fibroblasts, the accumulation of connective tissue and an irreversible alteration of the tissue architecture, leading to fibrosis and chronic lung disease.

1.9 Clinical Interventions

Recent clinical interventions have reduced the severity of acute RDS due to surfactant deficiency, but so far have had little impact on the outcome of chronic lung disease.

1.9.1 Exogenous Surfactant Therapy

Exogenous surfactant therapy has for the last 3 - 4 years been used as a routine rescue treatment for RDS. This has resulted in a decrease in the pulmonary complications of RDS, a reduced oxygen requirement and reduced ventilator pressure support for the initial stages of the disease. It has not contributed to reducing the numbers of infants developing chronic lung disease (Sinclair and Bracken.,1992). This may be a reflection of the increasing numbers of infants that would previously have died early from respiratory failure due to severe RDS, but with surfactant therapy now go on to develop chronic lung disease.

Exogenous surfactant is available in many forms e.g. Survanta (bovine), Curosurf (porcine), ALEC (synthetic) and Exosurf (synthetic). Curosurf and Exosurf were the two surfactants used by our unit during the study period of this thesis. Curosurf is derived from the minced lungs of pigs and contains both the lipid and protein parts of surfactant. Exosurf is synthetic and contains lipids but no proteins. Instead it contains two synthesised components hexadecanol and tyloxapol which mimic the proteins SP-B and SP-C, and which are essential if the surfactant is to form a surface film at the air/water interface.

There has been debate about the merits of natural versus synthetic surfactants; natural surfactants may be more immunogenic due to their proteins, but synthetic surfactants may not work as efficiently due to the lack of proteins. Halliday (1995) recently reviewed eight clinical trials of natural versus synthetic surfactant and concluded that there was reduced neonatal mortality and a reduced incidence of pulmonary air leaks, retinopathy of prematurity and chronic lung disease in the group receiving natural surfactant.

Surfactants have properties other than reduction of surface tension. They are important modulators of immune functions of the resident alveolar macrophages and because the aetiology of chronic lung disease involves a significant pulmonary inflammatory response it may be that surfactants can influence the inflammatory response. The role of normal surfactant proteins in immunomodulation has been discussed previously (see under " Surfactant p2"), and *in vitro* and animal studies of

Curosurf and Exosurf have shown them to have differing effects on immune responses.

Exosurf inhibits alveolar macrophage production of IL-1β, TNF-α and IL-6 (Thomassen *et al.*,1994) but not IL-8 (Thomassen *et al.*,1992) and may function as an antioxidant by scavenging oxygen radicals (Ghio *et al.*,1994). Curosurf in contrast inhibits monocyte TNF-α and superoxide anion production (Ciccimarra.,1994) and phagocytosis of *S. aureus* (Speer *et al.*,1991). Exogenous surfactant has been shown to promote endogenous surfactant production (Gerdes *et al.*,1992).

In rabbits decreased bactericidal killing by newborn alveolar macrophages is seen with both natural and synthetic surfactant (Sherman *et al.*,1988), but Exosurf limits the proliferation of group B streptococci in preterm rabbits more than Curosurf (Sherman *et al.*,1994). Curosurf treatment results in higher numbers of alveolar macrophages in bronchoalveolar lavage at days 5 and 7 but this does not correlate with outcome (Arnon *et al.*,1993).

1.9.2 Steroids

Maturation of the fetal lung is under corticosteroid control (Gross.,1990) and dexamethasone is given routinely in our unit in the ante-natal period for mothers threatening a premature delivery. It is hoped that this will promote lung maturation *in utero*, stimulate surfactant production, improve lung compliance and decrease the risk of respiratory distress syndrome (RDS).

In a meta-analysis of 12 clinical trials by Crowley *et al* 1990, administration of ante-natal steroids was shown to reduce RDS and the severity of RDS, and to reduce the incidence of intra-ventricular haemorrhage.

Clinical studies investigating dexamethasone treatment postnatally in preterm infants at risk for chronic lung disease have shown it improves surfactant function (Kari *et al.*,1994), reduces collagen synthesis (Co *et al.*,1993) and decreases levels of albumin and fibronectin (Watts *et al.*,1992a), in bronchoalveolar lavage fluid of treated infants. This suggests that the integrity of the alveolar capillary barrier is improved, surface tension is reduced and long term fibrosis avoided. Markers of lung inflammation,

neutrophils, neutrophil elastase, leukotriene B_4 and IL-1β (Groneck *et al.*,1993c, Murch *et al.*,1996b), TNF-a, MIP-1a and elastase (Murch *et al.*,1996b) and C5a (Groneck *et al.*,1993b) were also reduced in bronchoalveolar lavage.

Rastogi *et al* (1996) conducted a randomised double-blind placebo-controlled trial giving dexamethasone within 12 hours of birth and for 12 days to at risk infants (> 24 weeks gestation, < 1500g birthweight). The treatment group was weaned from the ventilator earlier and the incidence of chronic lung disease and death were significantly reduced compared to controls. Kari *et al* (1993) conducted a randomised double-blind placebo-controlled trial giving dexamethasone after 10 postnatal days in the same at risk group for seven days. The short term pulmonary outcome was improved in the treatment group, but not the longer term outcome of mortality, length of stay in hospital or oxygen requirement. A short course of dexamethasone (two treatments within the first 36 hours of life) will also reduce the short term ventilatory support but does not reduce the incidence of chronic lung disease (Sanders *et al.*,1994). Early and prolonged treatment would seem to be more beneficial in reducing the long term complications.

Dexamethasone has side-effects (Ng.,1993), though in the three studies above, the only side-effects shown was a higher incidence of hypertension in the studies by Kari and Sanders.

The mode of action of dexamethasone is not known, but as it is an anti-inflammatory agent it may improve outcome by suppression of the inflammatory response in the lungs of ventilated infants.

1.9.3 Antioxidants

There are relatively few studies which have investigated the administration of exogenous anti-oxidants and related them to outcome. Vitamin E was shown to be ineffective in reducing chronic lung disease (Saldanha *et al.*,1982), but recombinant superoxide dismutase has been reported to reduce the levels of inflammatory markers (albumin and elastase) in bronchoalveolar lavage fluid (Rosenfeld *et al.*,1996).

1.9.4 High Frequency Oscillatory Ventilation

Recent studies have focused on the use of high frequency oscillatory ventilation (HFOV) which accomplishes gas exchange at lower intrapulmonary distending pressure, so reducing the barotrauma of intermittent forced ventilation. Whilst this ventilator is still being investigated it has in early studies been shown to reduce the outcome of chronic lung disease, when used early and continuously in infants with RDS (Gerstmann *et al.*,1996; Clark *et al.*,1992). Our unit acquired HFOV recently and it was used only towards the end of this thesis study period. However preliminary data on a small cohort suggests that whilst death is delayed there is no reduction in overall mortality (Laing, 1997; personal communication).

1.9.5 Erythromycin

Erythromycin is one of the family of macrolide antibiotics which affects bacterial protein translocation at the ribosomal level. It is bacteriostatic at low concentrations and bactericidal at higher concentrations. It is readily internalised by phagocytes against a concentration gradient (Hand *et al.*,1983) and is not degraded by intracellular metabolism (Washington *et al.*,1985). Whilst the exact mechanism by which phagocytes internalise erythromycin is as yet unknown it is energy (Viggiano *et al.*,1994) and calcium ion dependent (Rowen *et al.*,1995) and phagocytosis of particles increases its intracellular concentration (Santoro *et al.*,1995). It is effective against the genital mycoplasmas (whereas gentamicin and penicillin are not) and is the treatment of choice in neonates (Waites *et al.*,1993). It is not used routinely in our unit.

The immunomodulating effects of erythromycin are currently under debate. Several clinical studies have shown low-dose long term erythromycin treatment to be effective in adult chronic airway diseases (Sawakai *et al.*,1986; Kudoh *et al.*,1987) where *Pseudomonas* infections persist. Its effectiveness possibly lies in its ability to reduce neutrophil recruitment into the lungs. This is supported by *in vitro* studies on the effect of erythromycin on neutrophil chemotaxis (Nelson *et al.*,1987; Esterley *et al.*,1990). Neutrophils show less elastolytic activity (Ichikawa *et al.*,1992) and

reduced phagocytosis (Hand *et al.*,1990). The mode of action of suppression of phagocytosis and chemotaxis may be related to disrupted mobilisation of calcium ions in the cells. It is known that calcium ions are important in microtubule assembly which is essential for PMN migration. Recently it has been suggested that erythromycin can perturb cAMP intracellularly (Aoshiba *et al.*,1997) promotes neutrophil apoptosis (Rossi *et al.*,1995). Apoptosis is the programmed cell death of neutrophils which are then phagocytosed by macrophages. The neutrophils invading the lung may therefore be programmed to die before any damage can be done.

Oishi *et al.*, 1994 evaluated IL-8 levels in bronchoalveolar lavage samples from adults with chronic airway disease and saw a reduction in IL-8 in the treatment group. They confirmed their findings using *in vitro* studies which showed erythromycin suppressed neutrophil, but not alveolar macrophage, derived IL-8. *In vitro* studies on the effect of erythromycin on cytokine production have shown that in response to LPS monocytes will increase IL-6 production (Bailly *et al.*,1991), but epithelial cells have their IL-6, IL-8 and IL-1β synthesis reduced (Khair *et al.*,1995). However, the method by which it affects the production of cytokines is as yet unclear.

Clinical studies using erythromycin in neonates for the eradication of the genital mycoplasmas in respiratory infections are few. Heggie *et al* (1994) treated infants with erythromycin after positive identification of the genital mycoplasmas from either CSF or bronchoalveolar lavage. They found no difference in the clinical outcome for colonised vs. non-colonised infants after treatment. However, the method they used for isolation of the genital mycoplasmas may have resulted in a large number of false negatives (Waites *et al.*,1995) and the mean start of treatment was 16.4 days after admission to their unit. It is possible the damage might already have been done by the infecting agent and subsequent inflammatory response.

1.10 Aims of this Thesis

i) To investigate the early inflammatory response both cellular and cytokine in bronchoalveolar lavage samples of the lungs of preterm infants and to relate this to outcome.

ii) To further investigate the genital mycoplasmas as a mechanism of stimulation of the inflammatory response, and to assess the effectiveness of erythromycin in reducing adverse outcome.

iii) To develop a cell based model of lung inflammation to better understand the interactions of lung epithelial cells, bacteria and mediators used in the clinical management of preterm infants.

iv) To compare a differential stain to an immunohistochemical stain for the identification of monocyte/macrophage cells in bronchoalveolar lavage samples.

2. Bronchoalveolar Lavage

2.1 Introduction

Bronchoalveolar lavage is a routine procedure in intubated infants as they can not cough to remove mucus. It involves the squirting of sterile saline down the endotracheal tube and then the insertion of a catheter to remove the saline and mucus by suction. It is performed routinely, requires no ethical permission and the sample collected can be analysed for cells and mediators. There are several protocols used for bronchoalveolar lavage which vary in volume of saline instilled and in number of lavages performed at sampling time. There is also debate about whether dilution of the epithelial lining fluid (ELF) by saline can be corrected for by using a reference protein. This makes comparison between studies difficult. The technique employed in our unit is constrained within clinical guidelines and these are discussed below, as is the reason for not using a reference protein to correct for dilution of the ELF.

2.1.1 Sample Size

In infants the lavage technique is blind i.e. the suction catheter is pushed down the endotracheal tube and suction is applied when resistance to the distal tip of the tube is felt by the operator. This means that the area being sampled can not specifically be known. A significant volume of aspirated sample is required to allow the measurement of several mediators (and also to ensure that there is enough sample to detect those mediators). For obvious reasons only small volumes of saline can be instilled into the bronchial tree and there is considerable loss of saline on aspiration. Some studies have used a fixed volume of saline regardless of infant size (Groneck *et al.*,1994a), whilst others use a volume related to infant size (Grigg *et al.*,1992). One way to increase sample volume is to sequentially sample the lungs by repeated bronchoalveolar lavage and to pool the aspirates. The first aliquots are known to sample trachea, with subsequent aliquots sampling more distal parts (Grigg *et al.*,1992). Although bronchoalveolar lavage is not harmful to infants, our clinical staff believe it is distressing and so sequential sampling was considered inappropriate. We chose 0.5 ml

saline for all infants, as this was a compromise between drowning and effective aspiration. As only a single sample was taken at each time point, the sample volume at each point was inevitably low. The recovered sample volume for each bronchoalveolar lavage sample was recorded.

2.1.2 Sample Timing

Times of sampling in the literature vary enormously between studies. In this study we felt that an initial sample taken as soon as possible after birth and prior to surfactant administration was important as a baseline. Infants are routinely sucked out on labour ward before intubation and their transfer to the Neonatal Intensive Care Unit (NICU). After arrival at the NICU a proper bronchoalveolar lavage was performed and the sample collected for processing. Samples were then collected as close to 24 hours intervals as possible when clinically indicated. Infants were sampled initially daily for as long as the infant was intubated, but after our pilot study and given that we wished to investigate the early inflammatory response we subsequently collected samples for the first five days of life only.

2.2 Markers of Dilution of Epithelial Lining Fluid

The recovered bronchoalveolar lavage fluid consists of epithelial lining fluid diluted in saline, and this contains cells, proteins and inflammatory mediators. It is difficult to know how much epithelial lining fluid has been recovered, but there have been suggestions that the dilution effect can be estimated by measurement of proteins in the epithelial lining fluid and by comparison of these with their concentration in plasma. This assumes that concentrations are the same in plasma and epithelial lining fluid and that they do not change during the disease process. Albumin (Reynolds.,1987), urea (Rennard *et al.*,1986) and the secretory component of IgA (Watts *et al.*,1992b) amongst others have been investigated.

2.2.1 Albumin

Albumin is present in the epithelial lining fluid at about 1/3 concentration of that in plasma due to oncotic pressure of most interstitial fluids (Walters *et al.*,1991), it increases in some disease states (Jones *et al.*,1990) and can be affected by treatment (Duddridge *et al.*,1990). It also increases in serum with postnatal age (Watts *et al.*,1995).

2.2.2 Urea

Kelly *et al* 1988 showed that the fluid dynamics during lavage are complex, and that during injection of fluid water passes from the bronchopulmonary segment into the blood or interstitium and at aspiration water is drawn from the plasma. Urea almost certainly moves into the lavage fluid from the plasma during the procedure by diffusion down a concentration gradient (Marcy *et al.*,1987).

2.2.3 Secretory Component of IgA

Watts and Bruce 1995 claim that secretory component in epithelial lining fluid is independent of capillary leak, gestational age and post-natal age during the first month of life. However, they only studied samples from day 2 onwards and Groneck *et al* 1994a, showed that secretory component was significantly lower on day 1 of life compared with day 3. It would therefore be of no use for our initial samples. Stoltenberg *et al* 1993 investigated post-mortem lung tissues and stained them for the cells expressing secretory component, the bronchiolar epithelium. Premature infants of <35 weeks gestation that only lived for 1 week postnatally had significantly less cells staining positive for secretory component than in mature infants with the same post-natal age.

It is known that secretory component expression is upregulated by several cytokines including TNF-α (Kvale *et al.*,1988) and TNF-α is detectable in many of the bronchoalveolar lavage samples of these infants (Murch *et al.*,1992).

2.2.4 Conclusions

It is clear from the literature that none of these proteins is suitable and that so many studies use different proteins or none at all shows there is as yet no consensus on what substance should be used as a reference for the dilution effect seen during lavage.

It was decided for the erythromycin trial (see "Chapter 5 p77") that urea measurements should be made on our samples because our sample size was too small for secretory component ELISA and the Department of Paediatric Biochemistry at RHSC was capable of measuring urea in 50 µl of sample. The results were to be compared both with and without the correction for urea.

2.3 Bronchoalveolar Lavage Method

Bronchoalveolar lavage was performed by instillation of 0.5 ml 0.9% sterile saline down the endotracheal tube. The ventilator was reconnected for 3-4 breaths and a suction catheter (6 FG) was pushed down the tube until resistance to the distal tip of the tube was felt. Suction was applied and the catheter removed. The sample was collected into a mucus trap (Vygon, UK) and stored at 4°C until processed.

2.4 Bronchoalveolar Lavage Sample Preparation

2.4.1 Introduction

There is no standardised method of sample preparation. We have attempted to develop a technique which allows us to conduct all the studies we wished to do without significantly altering the sample.

2.4.2 Time to Processing

There are conflicting data on how long samples should be stored at 4°C before processing (Schumann *et al.*,1992), with some reports that samples are satisfactory up to 4 hours (Linder *et al.*, 1988) and others suggesting that cells are lost within 30 minutes. We kept the time to sample processing to a minimum and there was always

someone on-call to process the sample within 4 hours of it being collected. The average time to sample processing for samples for each study is given.

2.4.3 Fixatives

The use of fixatives to stabilise the sample for transport was inappropriate as we wished not only to measure inflammatory mediators but also perform immunocytochemistry on the cells. The sample also only had a five minute journey to the lab and was then processed immediately so it was not considered to be necessary.

2.4.4 Mucus

Mucus may affect the sample by introducing a population of inflammatory cells as well as creating a poor staining environment (Schumann *et al.*,1992). An experiment to remove mucus from four samples was performed and the effect on the cell populations investigated.

Methods

Four samples were collected and processed as described above. The sample was strained through a sterile strainer (Becton Dickinson, Cat No. 2350, Pore size 70μm) with 1 ml of RPMI-1640 medium (PFC, Edinburgh) and the fluid collected into a 1.5 ml sterile tube. The mucus was removed by inverting the strainer and flushing 1 ml of RPMI-1640 in the opposite direction collecting the mucus containing fluid into a sterile 1.5 ml tube. Both samples were spun separately at 140g for 15 minutes at 4°C (Jouan, CR55) and the supernatant discarded. The cell pellets were both resuspended in 200μl medium and cell counts made in a haemocytometer (Neubauer). Viability was assessed by trypan blue exclusion. The cell concentration was adjusted to 4×10^5 cells/ml and 100μl was added to a cytospin slide and spun at 1000 rpm for 3 minutes (Shandon, Cytospin 3). The slide was air dried, stained differentially (Dade, Diff-Quik) and 300 cells were counted.

Results

Table 2-1: Mucus Extraction Experiment Results.

Cell percentages with mucus removed and not removed from bronchoalveolar lavage samples.

Cells	Mucus Removed	Mucus Remaining	Paired t-test
% Viability	83	68	
	75	100	
	88	83	
	87	56	p = 0.62
% Neutrophils	70	71	
	80	79	
	84	93	
	62	76	p = 0.20
% Macrophages	23	26	
	18	18	
	16	3	
	35	23	p = 0.27
% Epithelial cells	7	3	
	3	4	
	0	4	
	3	3	p = 0.89

Discussion

There was no significant differences between the counts using a paired t-test nor when the values are log transformed (data not shown). Removal of mucus makes the sample easier to process and the differential stain clearer therefore our samples were strained before analysis.

2.4.5 Removal of Supernatant

The sample volume was estimated visually and the mucus was removed by straining the sample through a sterile cell strainer (Becton Dickinson, Cat.No. 2350, Pore size 70μm). The sample was flushed through with a small amount of RPMI-1640 if necessary, to make the volume of sample up to a maximum of 0.5 ml. Any extra

volume added to the sample was noted. The sample was placed into a sterile, labelled 1.5 mls tube and spun at 140g for 10 minutes at 4°C. 50µl of the supernatant was aliquoted into sterile, labelled 0.5 ml tubes and frozen at -70°C immediately.

2.4.6 Blood Stained Sample

If the sample was blood stained then after removal of the supernatant for freezing 500µl of distilled sterile water was added and this was left for approximately 1 minute to lyse the red blood cells. 500µl of medium was added and this was spun at 140g for 10 minutes at 4°C. The supernatant was discarded and the cells processed as below.

2.4.7 Cell Preparation

After 100µl of medium had been added the cells were resuspended by gently flicking the bottom of the tube.

The haemocytometer was prepared by wiping with alcohol and placing the coverslip over the counting chamber so that interference rings could be seen. A viability count was done by exclusion of trypan blue. The cell concentration was adjusted to 4×10^5 cells/ml in medium and 100µl was added per slide in a cytospin and spun at 1000 rpm for 3 minutes. Slides were allowed to air dry and one slide was immediately stained for differential cell count. The other slides were left overnight at room temperature before being frozen at -20°C with desiccant for later immunocytochemical stain (see "Chapter 6 p117").

3. Development of Interleukin-8 Radioimmunoassay

3.1 Introduction

The principles of radioimmunoassay are relatively simple. A fixed concentration of antibody is incubated with varying concentrations of antigen. A fixed concentration of radioactively labelled antigen (tracer) is then added which binds any remaining antibody sites and will compete within the non-radioactively labelled antigen until an equilibrium is reached. Any unbound antigen is removed. The bound fraction is counted in a gamma counter and the amount of labelled antigen counted is inversely related to the concentration of known antigen. This gives a standard curve from which unknown antigen values can be calculated.

To develop a suitable assay for measuring unknown antigen concentrations requires several things;

1. a specific antibody and the calculation of its concentration
2. a labelled antigen which can be measured precisely
3. a suitable standard from which unknown concentrations can be calculated by comparison
4. a means of efficiently separating free from bound antigen.

A suitable antibody must be both specific for and bind strongly to the antigen. The primary IL-8 antibody used in this study was raised in a rabbit and was a gift. It had previously been used in a radioimmunoassay (Kelly *et al.*,1994) and its characteristics are discussed below. Commercially available standard of high purity is readily available and this was labelled with iodine-125 (^{125}I) in-house for our tracer.

The separation of bound from free antigen is necessary to allow calculations about the unknowns in our samples. There are several methods employed but the most common and simplest is the use of a second antibody directed against the first antibody. This primary-secondary-antigen complex can then be separated by centrifugation from any unbound antigen. A donkey anti-rabbit antibody was available to us free of charge and

will bind specifically to the primary rabbit anti-IL-8 antibody. Precipitation of the antigen-antibody complex only occurs at high antigen-antibody complex concentrations and so a carrier protein is used from the species in which the primary antibody was raised. Normal rabbit serum in a solution of assay buffer and polyethylene glycol was used as this is quick, specific and simple allowing reproducibility and increased sensitivity. The separation would ideally be 100% but in practice this never occurs. A portion of the free tracer behaves as if it were bound and this is measured as the non-specific binding (NSB). There may be "free" tracer caught up in the antigen-antibody complex, tracer impurities or it may adsorb to the assay tubes. The NSB is measured by adding only assay buffer and tracer to a tube and treating it as any other sample. It is then deducted from the counts before any calculations are made.

The values of the unknowns are calculated by comparison to a set of standard known values. The tracer concentration is fixed and varying amounts of the unlabelled standard are added. The value at which no unlabelled standard is added represents the maximum binding (B_0) and all subsequent values are expressed as a percentage of this. The percentage of tracer bound is progressively reduced with increasing concentrations of standard added. A straight line representing the range of the assay occurs between the extremes of the curve and unknown values can be calculated from the curve.

3.2 Materials

3.2.1 Antibody

The IL-8 antibody was a gift from Dr Rodney Kelly (Medical Research Council Reproductive Biology Unit, Edinburgh). It was supplied as a freeze-dried powder which was reconstituted in assay buffer, aliquoted and frozen at -20°C.

It was shown to have no cross-reactivity with IL-1, IL-2, IL-6 and IL-10 (Kelly *et al.*,1994).

3.2.2 Standard

The IL-8 standards and quality controls came from NIBSC (batch 89/520) and R&D Systems, Massachusetts. The NIBSC standard was diluted to 40ng/ml and frozen at -20°C in 500µl aliquots. The R&D standard was diluted to 50µg/ml and stored at -20°C in 10µl aliquots.

3.2.3 Iodination of IL-8

Iodinated IL-8 tracer was supplied by Dr Kelly.

Iodination and purification were carried out as follows;

Gloves were washed to remove any starch and safety procedures for handling radioactive materials were followed.

1. 0.75mCi (7.5µl) ^{125}I was put into an iodination tube containing 10µg IL-8.
2. 10µl chloramine T [5mg/ml chloramine T in 0.5M iodination buffer pH 7.4 (oxidising agent)] was added and vortexed.
3. This was incubated for 20 seconds then 20µl sodium metabisulphite [7mg/ml sodium metabisulphite in 0.5M iodination buffer pH 7.4 (reducing agent)] was added and vortexed.
4. 0.5ml 2 x iodination buffer (with detergent and bovine serum albumin) was added to the iodination tube and mixed.
5. A PD-10 Sephadex G25 column was washed with 2 x iodination buffer (with detergent and BSA).
6. Using a glass pipette the iodination mixture from (4) was added to the column.
7. 3ml non stick buffer was allowed to drip through the column twice and this was collected and used as labelled IL-8.

The labelled IL-8 was stored at 4°C until use.

3.3 Optimisation of Antibody Concentration and Assay Volumes

Typically a bronchoalveolar lavage would yield only 100 to 200µl of sample and in order to carry out the several measurements we wished to perform it was necessary to dilute the sample up to 500µl. These small sample volumes meant that we needed to develop a sensitive assay and several experiments were done to optimise this.

The antibody concentration chosen is usually the amount of antibody which binds 50% of the antigen. At this concentration the addition of further antigen leads to an increase in the free compared to the bound fraction. As the B0 decreases the sensitivity of the assay increases but precision can be lost and there is a lower limit of 20% beyond which precision is too variable (Burdon and van Knippenberg.1987). We wished to improve the sensitivity of the assay and so were aiming for about 25-30% binding with no loss in precision.

3.3.1 Methods

Antibody dilutions

The assay previously described (Kelly *et al.*,1994) used 1:20,000 initial dilution of IL-8 antibody. When referring to antibody dilutions in this thesis all references are to initial dilutions unless otherwise stated. A series of antibody dilutions were made in assay buffer at 1:10,000, 1:20,000, 1:30,000, 1:40,000 and 1:50,000 dilutions.

Tracer

5000 counts per minute (cpm) in each of the different volumes.

Table 3-1: Volumes of assay constituents.

IL-8 Antibody	Tracer	Assay buffer
50µl	50µl	50µl
50µl	50µl	100µl
50µl	50µl	200µl
100µl	100µl	100µl
25µl	25µl	50µl
25µl	25µl	100µl
25µl	25µl	200µl

These volumes were made up in duplicate and incubated for 48 hours at 4°C. 200µl of the precipitating antibody solution was then added to each tube after a further incubation for 1 hour at 4°C, separation of bound and free were effected by centrifugation at 3900g (Jouan, CR422). The supernatant (containing the free fraction) was aspirated and the pellet (containing the bound fraction) counted in a gamma counter (Packard Bell).

3.3.2 Table of Results

Table 3-2: Values are expressed as % of the total counts.

Antibody **Tracer** **Buffer (sample)**	**50µl** **50µl** **50µl**	**50µl** **50µl** **100µl**	**50µl** **50µl** **100µl**	**100µl** **100µl** **100µl**	**25µl** **25µl** **50µl**	**25µl** **25µl** **100µl**	**25µl** **25µl** **200µl**
NSB	5.2	2.1	1.8	2.2	1.6	2.0	2.9
1:10,000	38	35	35	39	37	38	37
1:20,000	34	32	30*	36	35	35	34
1:30,000	33	33	31	34	30*	29*	24*
1:40,000	33	31	31	34	29*	28*	24*
1:50,000	31	28*	28*	31	25*	25*	21

3.3.3 Discussion

Several values (*) are within the 25-30% range, with the majority at tracer and antibody volumes of 25µl, but variable sample volume. At these volumes an antibody concentration less than 1:30,000 would appear best. The intra-assay variability with these parameters was less than 10% for all samples (results not shown) so precision is not lost.

3.4 Sample Volumes

The sample volumes in the assay needed to be as small as possible to allow other measurements from the small lavage samples. The improvement in sensitivity of the assay at lower B0 must be offset with the loss of accuracy that can occur at lower volumes and concentrations of the other constituents.

3.4.1 Methods

Antibody

Antibody was diluted to 1:30,000 and 1:60,000 in assay buffer.

Tracer

The tracer was diluted to 5000cpm in assay buffer.

Standard

IL-8 (NIBSC) standard was diluted in assay buffer to give a range of standards from 5 ng/ml to 10 pg/ml concentrations.

Tubes of standard and antibody, as in
Table 3-3: Volumes of Assay Constituent below, were prepared in triplicate and incubated for 24 hours at 4°C. Tracer was then added to each and further incubated for another 24 hours at 4°C, before separation.

Table 3-3: Volumes of Assay Constituents

IL-8 Antibody	Tracer	Standard
50µl	50µl	100µl
50µl	50µl	200µl
25µl	25µl	50µl
25µl	25µl	100µl
25µl	25µl	200µl

200µl of the precipitating antibody solution was added to each tube and after a further hour at 4°C separation of bound and free was achieved by centrifugation at 3900G (Jouan, CR422). The supernatant was aspirated and the pellet counted in a gamma counter (Packard Bell).

3.4.2 Results and Discussion

Table 3-4: Antibody concentration at 1:30,000. Results expressed as % bound ÷ B0.

Antibody Standard Tracer	50µl 100µl 50µl	50µl 200µl 50µl	25µl 50µl 25µl	25µl 100µl 25µl	25µl 200µl 25µl
NSB (%)	1.6	1.6	2.0	2.1	2.1
B0	29	27	29	24	21
10 pg/ml	100	99	95	99	94
50 pg/ml	98	88	93	88	75
100 pg/ml	88	70	82	70	54
500 pg/ml	47	29	45	29	19
1000 pg/ml	24	15	23	14	13
5000 pg/ml	11	12	11	8	5

Table 3-4 shows the results for the antibody concentration 1:30,000. There is good reproducibility in the triplicates (results not shown), with low NSB in all sample volumes. The value for B0 is between 25-30% for all but the 200µl sample volume

which is approaching the 20% threshold. At antibody and tracer volumes of 50µl a sample volume of 200µl gives good discrimination at the sensitive end of the range (10 - 50pg/ml), but 200µl is a very large sample size for our intentions and the assay would need to be very sensitive if we were to dilute the samples to this value. At antibody and tracer volumes of 25µl there is better discrimination at the sensitive end of the range for the sample volume 100µl compared to 50µl (see Graph 3.1) and in fact the sensitivity is comparable to the previous 200µl sample volume.

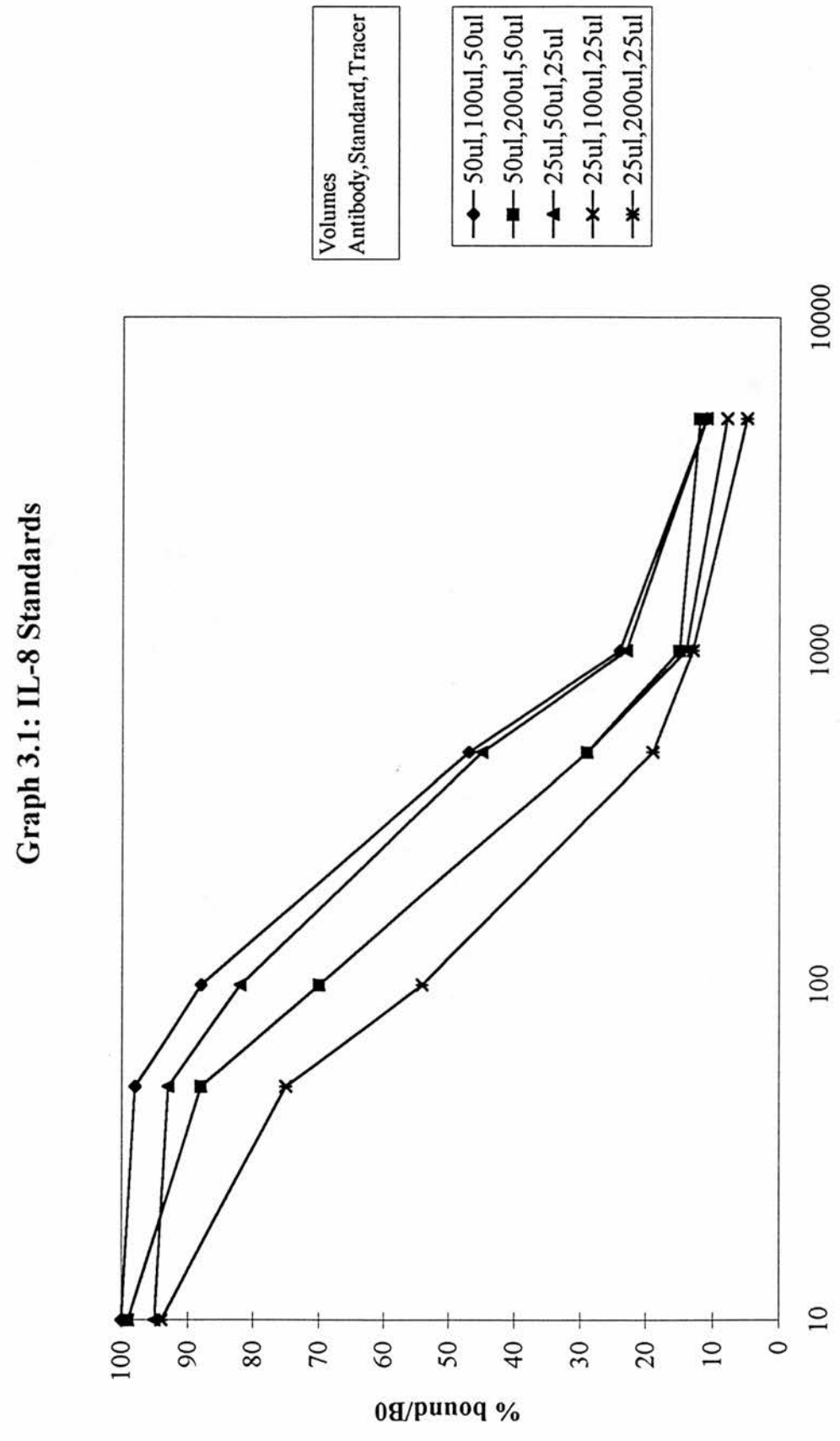
Table 3-5 shows the results for 1:60,000 antibody dilution. The B0 is below the threshold for all these samples.

Table 3-5: Antibody concentration at 1:60,000. Results expressed as % bound ÷ B0.

Antibody **Standard** **Tracer**	**50µl** **100µl** **50µl**	**50µl** **200µl** **50µl**	**25µl** **50µl** **25µl**	**25µl** **100µl** **25µl**	**25µl** **200µl** **25µl**
NSB	1.7	2.0	1.9	3.1	1.8
B0	20	17	19	17	13
10 pg/ml	94	89	92	84	83
50 pg/ml	82	67	79	68	52
100 pg/ml	65	43	64	39	14
500 pg/ml	28	15	26	10	0
1000 pg/ml	15	7	18	7	0
5000 pg/ml	7	2	9	2	0

3.4.3 Conclusions

Based on the results from these two experiments sample volumes will be 100µl and the other assay constituents will be 25µl.



Graph 3.1: Four IL-8 standard curves using different assay volume constituents.

3.5 Evaluating the In-House Tracer

The in-house labelling and purification of tracer is a cheap and reliable way of producing labelled antigen, but may not produce as high quality tracer as those commercially available. Commercially available tracers may have a more efficient iodination procedure i.e. less unlabelled IL-8 in the tracer and less impurities. Both of these will significantly reduce the non-specific binding and improve assay sensitivity. IL-8 has a tendency to dimerise in storage and so it was important to assess the shelf-life of the tracer.

A commercially available tracer (Amersham) was bought to compare to the in-house tracer. This was expensive and would have needed to significantly improve the sensitivity to be viable for our project.

A series of identical standard curves was performed over successive weeks to test the tracer shelf-life.

3.5.1 Methods

Standard

Standard (NIBSC): 5ng/ml to 5pg/ml diluted in assay buffer.

Antibody

IL-8 Antibody diluted in assay buffer to 1:20,000 and 1:30,000.

Tracer

Tracer from Amersham and in-house both diluted in assay buffer to 15000 cpm.

Volumes

25µl tracer + 25µl antibody + 100µl standard

Antibody and standard were added to triplicate tubes and incubated overnight at 4°C. The tracer was then added and the tubes left to incubate at 4°C for a further 24 hours. 200µl of the precipitating antibody solution was added to each tube and after a further

hour at 4°C separation of bound and free was achieved by centrifugation at 3900g. The supernatant was aspirated and the pellet counted in a gamma counter.

3.5.2 Results and Discussion

Table 3-6: Results of In-House vs. Commercial Tracer

Results expressed as % bound ÷ B0. Am = Amersham tracer; IH = in-house tracer.

Antibody Dilution	**1:20,000**		**1:30,000**	
Tracer	**Am**	**IH**	**Am**	**IH**
NSB	1.5	2.0	1.3	2.0
B0	50	45	33	33
5 pg/ml	97	98	91	100
10 pg/ml	96	97	86	96
50 pg/ml	81	83	79	76
100 pg/ml	68	75	61	62
500 pg/ml	28	25	26	24
1000 pg/ml	18	17	18	16
5000 pg/ml	7	8	7	9

Table 3-6 shows the results of the in-house vs. the commercial Amersham tracer. As expected the NSB was slightly lower for the Amersham tracer. At 1:20,000 dilution the B0 is too high and the sensitivity is lost. At 1:30,000 dilution of the antibody Amersham tracer gives better sensitivity at 5-10 pg/ml compared to the in-house tracer which can not discriminate between these two points, but does discriminate between 10-50 pg/ml well.

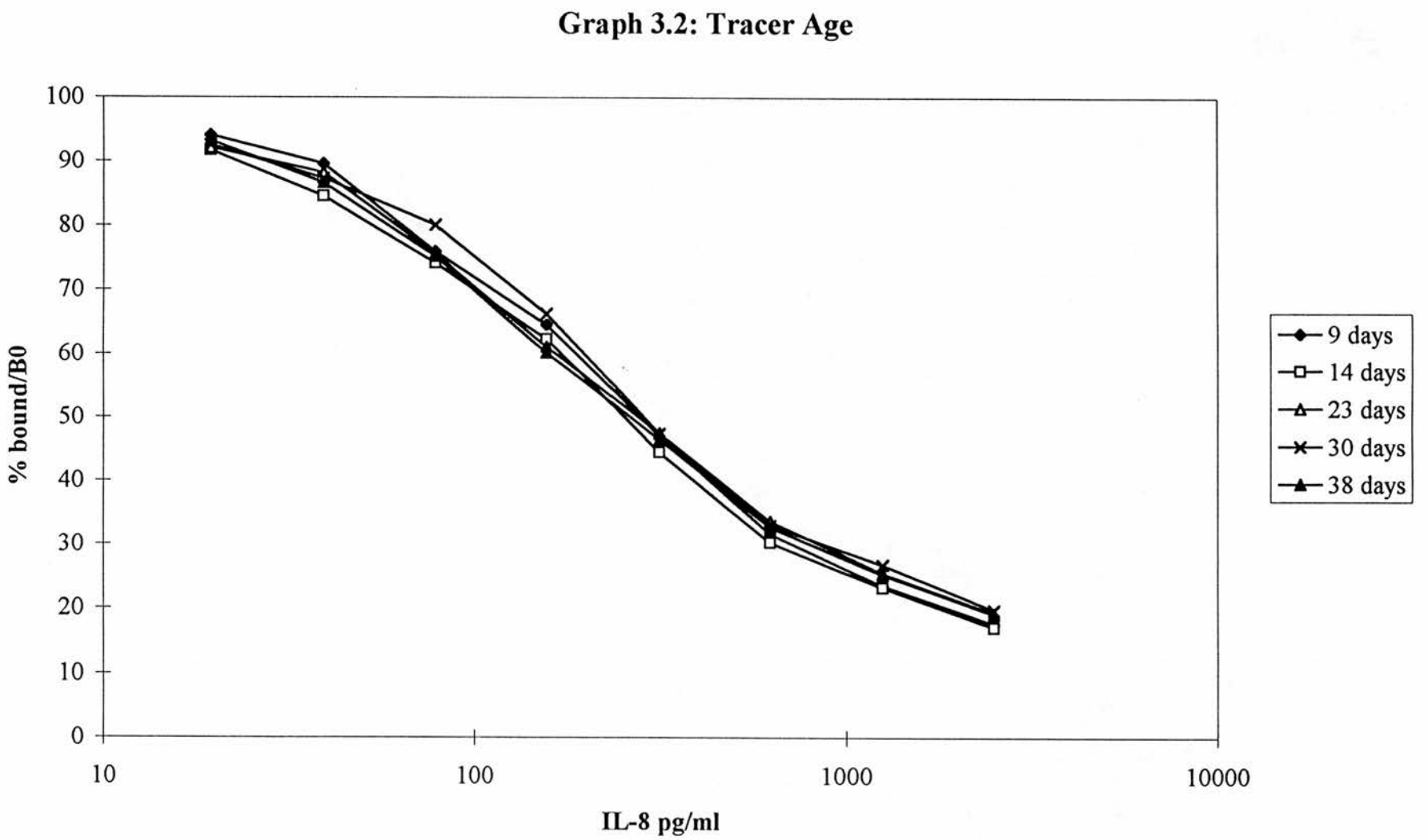
Although there is slightly better sensitivity with the Amersham tracer, this is not enough to warrant the expense of it and from previous studies measuring IL-8 the in-house tracer will be sensitive enough.

Table 3-7 show the results for total counts, NSB and B0 for standard curves using the same batch of in-house tracer over a period 38 days. The B0 decreases to 21% which is the lower limit of practical use, but the NSB remains essentially the same. Graph

3.2 plots the standards from these curves (expressed as %B0). There is very little difference between the standard curves over the 5 week period. The tracer will not be used past 5 weeks of age.

Table 3-7: Results for In-House Tracer over Time.

Tracer Age (Days)	Tc	NSB (%Tc)	B0 (%Tc)
9	17181	265.8 (1.55)	5362 (31.21)
14	14435	226.3 (1.57)	4036 (27.96)
23	16185	285 (1.76)	4682 (28.93)
30	17460	307.9 (1.76)	4418 (25.3)
38	16488	226.2 (1.37)	3479 (21.1)



Graph 3.2: Five identical standard curves using tracer of different ages

3.6 Equilibrated vs. Dis-equilibrated Assay

A radioimmunoassay relies on the reaction between the binding of the antibody and antigen and the formation of an equilibrium between the bound antigen-antibody complex and the free constituents. Each reaction (the standard + antibody, and the standard-antibody + labelled standard) requires time to achieve equilibrium and whilst it may be beneficial to have short incubation times to lessen the overall assay time, this may also decrease sensitivity and precision. An equilibrated assay involves the introduction of all constituents to the tube at once, whereas the dis-equilibrated assay involves each step in the assay having separate overnight incubations.

3.6.1 Methods

Standard

Standard (NIBSC): 5 ng/ml to 5pg/ml diluted in assay buffer.

Antibody

IL-8 Antibody diluted in assay buffer to 1:30,000.

Tracer

In-house tracer diluted in assay buffer to 15000 cpm.

Volumes

25µl tracer + 25µl antibody + 100µl standard

For both assays the tracer, standard and antibody were added to ten replicates.

For the equilibrated assay they were incubated for 48 hours at 4°C.

For the dis-equilibrated assay standard and antibody were incubated for 24 hours at 4°C and then tracer was added for a further 24 hours incubation at 4°C.

For both assays 200µl of the precipitating antibody solution was added to each tube and incubated at 4°C for 1 hour before being spun at 3900G. The supernatant was aspirated and the pellet counted in a gamma counter.

3.6.2 Results and Discussion

The reproducibility (% cv) was excellent for both experiments (Table 10-1 and Table 10-2 in Appendix B) with only one value above 6%. This was the NSB which had one value well above the rest. Graph 3.3 shows a comparison of the two curves plotted with % cv as the error bars. The equilibrated assay does not discriminate as well as the dis-equilibrated at the lower end of the standard curve (19.5 - 39.1 pg/ml) and so sensitivity appears to be lost when the reactions are not allowed time to come to equilibrium individually. The dis-equilibrated assay will be used.

Graph 3.3: Dis-equilibrated vs Equilibrated IL-8 Assay

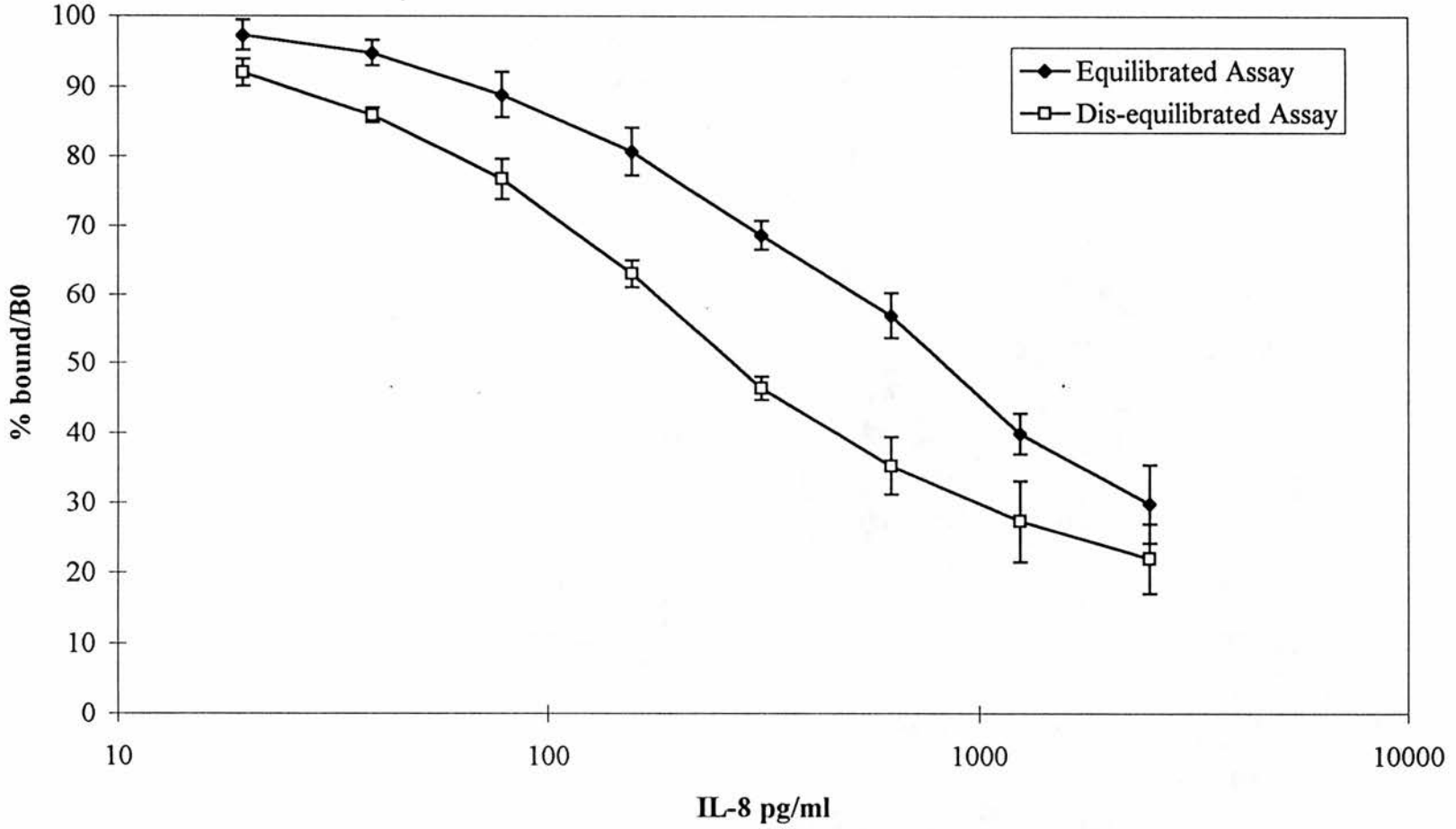


Figure 3.3: Identical IL-8 standard curves using a dis-equilibrated or equilibrated assay. Error bars are % cv of ten replicates.

3.7 Specificity

This antibody has been previously tested for its cross-reactivity with other related proteins to IL-8 (see "Materials p38"). However, it is also necessary to ensure that other substances in the bronchoalveolar lavage samples do not interfere with the assay and therefore affect the results. The non-specific binding gives us the interference due to any of the assay constituents, however the samples are tracheal secretions and the degree to which they interfere must be known. Serial dilutions of tracheal aspirate samples were prepared and assayed and the resulting curve compared to the standard curve to ensure parallelism.

3.7.1 Methods

Standard

Standard (NIBSC): 5 ng/ml to 5pg/ml diluted in assay buffer.

Antibody

IL-8 Antibody diluted in assay buffer to 1:30,000.

Tracer

In-house tracer diluted in assay buffer to 15000 cpm.

Volumes

25µl tracer + 25µl antibody + 100µl standard or sample

Sixteen tracheal aspirate samples TA1 to TA16 were diluted in assay buffer over 4 serial 1:5 or 1:2 dilutions.

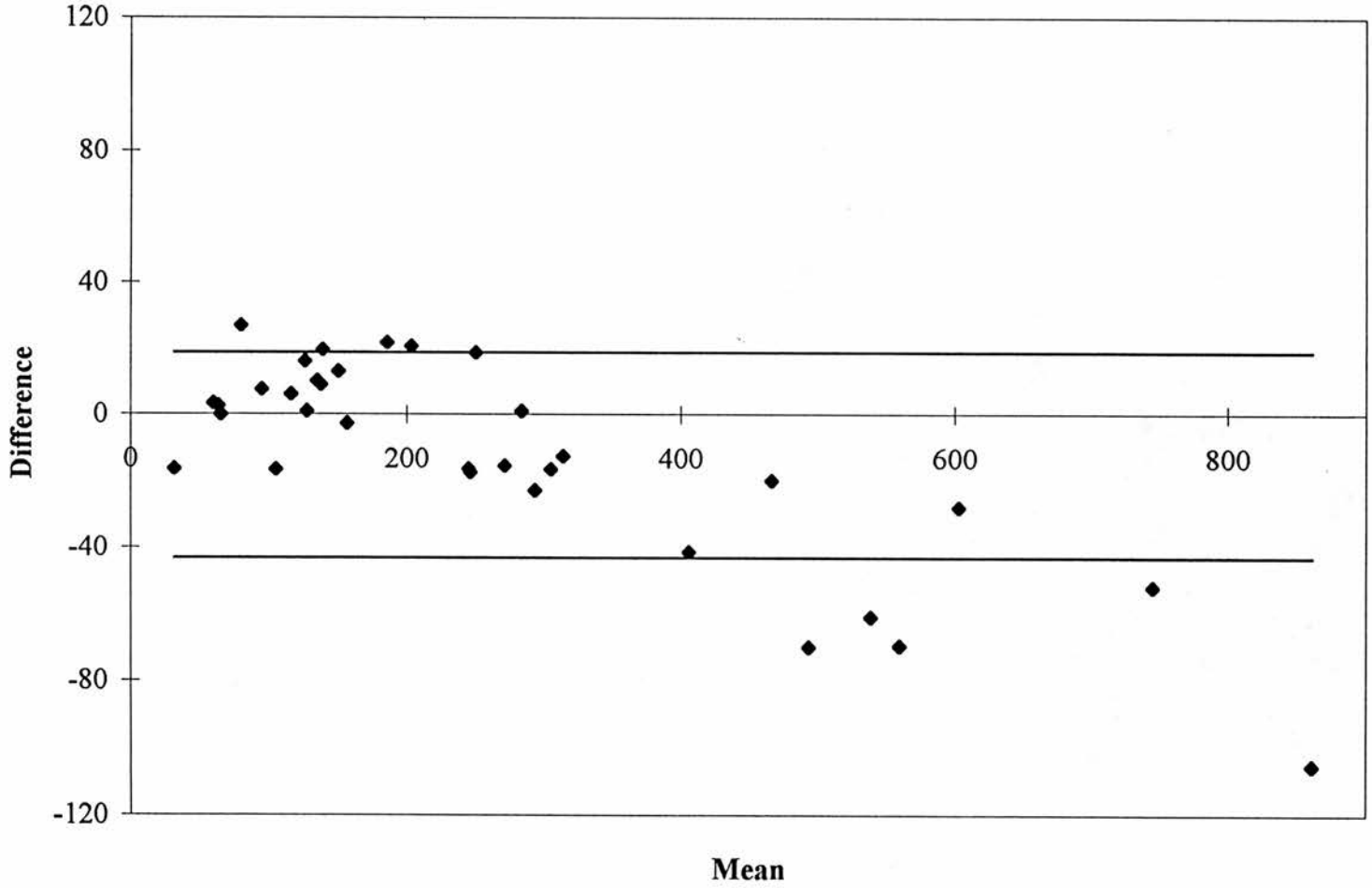
Standard or sample and antibody were added to triplicate tubes. They were incubated for 24 hours at 4°C. Tracer was added and incubated for another 24 hours at 4°C. 200µl of the precipitating antibody solution was added to each tube and incubated at

4°C for 1 hour before being spun at 3900g. The supernatant was aspirated and the pellet counted in a gamma counter.

3.7.2 Results

Table 10-3, Appendix B gives the results as the measured value and the expected value. If the samples dilute in parallel to the standard then we can predict the expected value by dividing the first measured value by the dilution factor. The results can be plotted using a Bland-Altmann plot (Graph 3.4) which has the mean of the expected and observed values on the x-axis vs. the differences of the expected and observed.

Graph 3.4: Bland -Altmann Plot for IL-8 Sample Dilutions



Graph 3.4: Plot of the mean of the observed and expected values vs the difference between the values. The dotted lines represent 1 standard deviation

3.7.3 Discussion

Nearly all the values lie within one standard deviation of the mean of the differences. The correlation coefficient is 0.996 and the antibody would appear to be binding specifically to the IL-8 without any interference from the sample matrix.

3.8 Comparison of NIBSC and R&D Standards

The NIBSC Standard was used in development of the assay, but for routine use IL-8 was purchased from R&D Systems, Massachusetts. The two standards were compared to ensure that the both standards measured equivalently.

3.8.1 Methods

Standard

Standard (NIBSC): 2.5ng/ml to 19.5 pg/ml diluted in assay buffer.

Antibody

IL-8 Antibody diluted in assay buffer to 1:30,000.

Tracer

In-house tracer diluted in assay buffer to 15000 cpm.

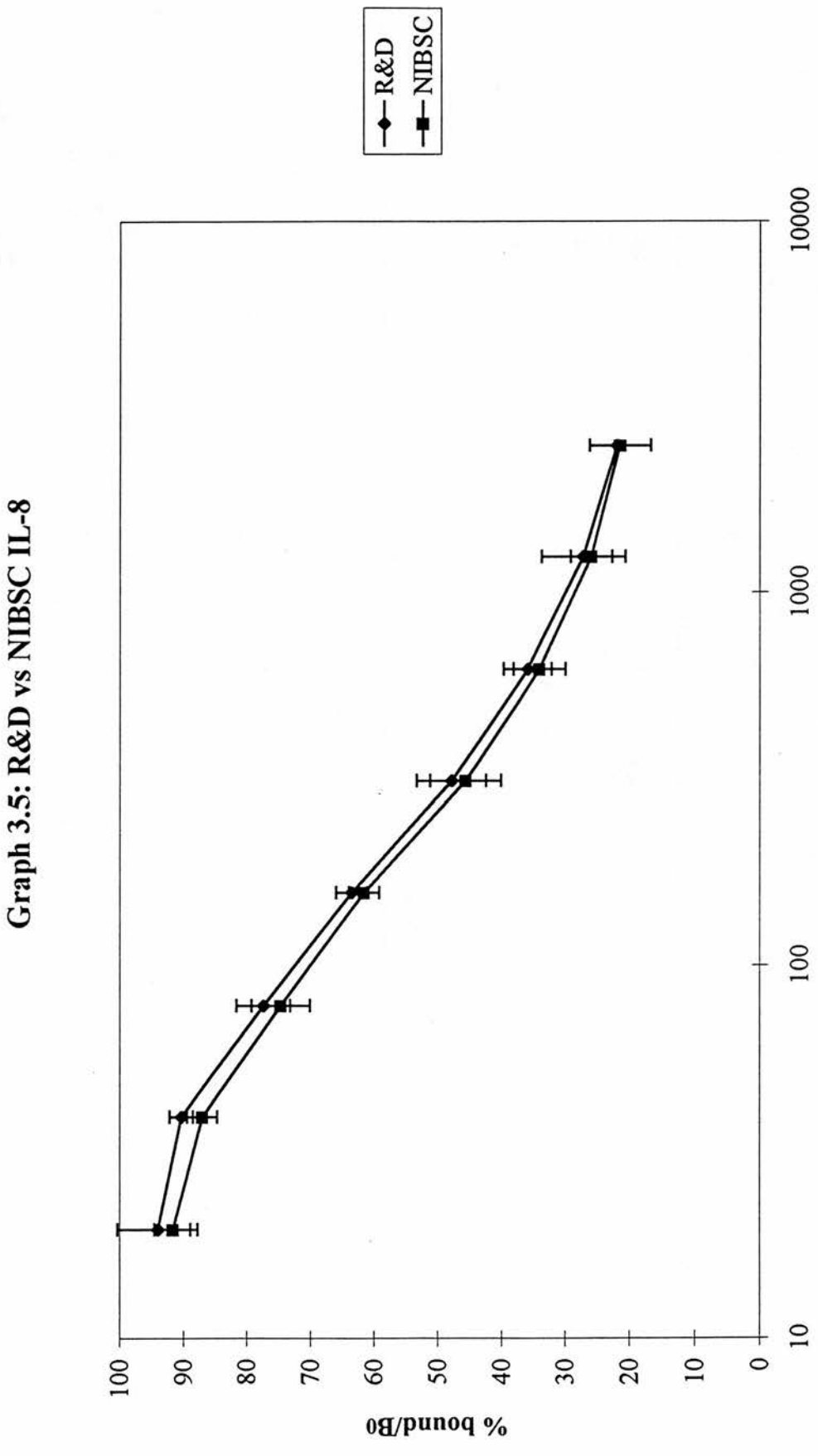
Volumes

25µl tracer + 25µl antibody + 100µl standard or sample

Antibody and standard were added to ten replicate tubes for each standard and incubated overnight at 4°C. The tracer was then added and the tubes incubated at 4°C overnight. 200µl of the precipitating antibody solution was added to each tube and incubated at 4°C for 1 hour before being spun at 3900g. The supernatant was aspirated and the pellet counted in a gamma counter.

3.8.2 Results and Discussion

Table 10-4 and Table 10-5 (in Appendix B) show the results for both curves. Percent cv are <7% for all replicates except the NSB and they are below 12%. Graph 3.4 shows both curves plotted against each with % cvs as error bars. There is no difference between the two standard curves and reproducibility is excellent for both.



Graph 3.5: Two identical IL-8 standard curves using IL-8 from different sources.

3.9 Quality Controls

Three quality controls of IL-8 (NIBSC) were used in 10 assays at approximately 20, 50 and 80 % binding, the straight part of the standard curve. Each was made in one batch in RPMI-1640 medium (PFC, Edinburgh) and frozen at -20°C as aliquots ready for use. The RPMI-1640 was used as the samples were to be diluted in this medium after collection.

3.9.1 Results

Table 3-8: Quality Control Values for 10 IL-8 Assays.

Quality Control Value	**800**	**150**	**30**
	759	153	30
	732	160	33
	870	190	38
	725	140	33
	860	122	29
	815	146	33
	875	161	32
	893	182	33
	720	180	26
	848	138	NA
Mean	809.7	157.2	31.9
Standard Deviation	68.9	21.8	3.3
%cv	8.5	13.9	10.5

3.9.2 Discussion

Inter-assay variation is < 14% based on these quality controls. Intra-assay variation is calculated for each standard and sample replicates by the Gamma-Counter and any coefficient of variation >5% is flagged and discarded. These samples are then re-tested where possible.

3.10 Final Assay

Samples were diluted appropriately in assay buffer and 100µl was added to duplicate tubes. 25µl of rabbit anti-human IL-8 diluted in assay buffer to 1:30,000 was added and incubated for 24 hours at 4°C. 25µl of ^{125}I labelled human IL-8 (R & D Systems) was added to all tubes and incubated for a further 24 hours at 4°C. 200µl of precipitating antibody solution was then added to each tube and incubated for 1 hour at room temperature. The tubes were then spun at 3900g for 20 mins at 4°C and the supernatants aspirated. The tubes were counted in a Canberra-Packard gamma counter. An eight point standard curve (range 5µg/L - 50ng/L) was included as were three quality controls (see "Section 3.9: Quality Controls").

The assay included three control samples as shown in Table 3-9 below to allow calculation of results

Table 3-9: Three Control Samples for IL-8 Radioimmunoassay.

	Tracer	Assay Buffer	Antibody
Total count (Tc)	25µl	-	-
Non specific binding (NSB)	25µl	125µl	-
B0	25µl	100µl	25µl

Results were expressed as percentage of the reference (B0) bound and were calculated from the standard curve.

4. Pilot Study

4.1 Introduction

Donnelly *et al* (1993) reported that the concentration of the neutrophil activating and chemotactic cytokine, interleukin-8 (IL-8), was greater in bronchoalveolar lavage samples from trauma patients who went on to develop adult respiratory distress syndrome (ARDS) than from patients who did not. The IL-8 could be detected on admission and before lung damage was clinically evident.

It is now recognised that there is a significant pulmonary inflammatory reaction in the development of chronic lung disease in premature infants (Robertson.,1989). Many studies have reported the presence of large amounts of neutrophils in bronchoalveolar lavage samples from premature intubated infants, (Ogden *et al.*,1984; Merrit *et al.*,1983) and recently Groneck (1993a) showed that IL-8 was the major chemotactic factor in the lungs of infants at risk for chronic lung disease.

Our aim was to measure the early inflammatory response, illustrated by IL-8, TNF-α and immune cells in bronchoalveolar lavage samples, in infants at risk of developing chronic lung disease.

4.2 Study Group

This was a pilot (prospective cohort) study in a tertiary referral neonatal unit for the south-east of Scotland. Infants of $\leq$ 30 weeks gestation intubated and ventilated immediately at birth were enrolled on the study. Our definition of chronic lung disease was any ventilated infant still in supplemental oxygen at 28 days of age with radiological abnormalities on chest x-ray.

4.3 Methods

4.3.1 Bronchoalveolar Lavage Samples

Details of the bronchoalveolar lavage technique are given in Chapter 2. In brief, after instillation of 0.5mls saline down the endotracheal tube a catheter was inserted and suction applied. The sample was collected in a mucus trap and placed in the fridge until assay. The mean (95% confidence interval) for sample volume recovered was 104.5 (82 - 127µl). The mean (95% confidence interval) time until assay was 1.5 hours (1.1 - 1.9) hours. The first sample was taken as soon as possible and always before surfactant administration. Subsequent samples were taken daily for the first three days for cytokine and cell measurements. Bronchoalveolar lavage samples were also sent daily for routine bacteriology.

4.3.2 Sample Preparation

This was as previously discussed (see "Chapter 2 p30"). In brief, the sample was flushed from the mucus trap using RPMI-1640 medium (SE-BTS, Edinburgh) and mucus was removed by straining the sample through a cell strainer. The cells were removed by centrifugation at 400g for 10 minutes. The supernatant was aliquoted and immediately frozen at -70°C. The cell pellet was resuspended in RPMI-1640 medium and the cells counted and the viability assessed by trypan blue exclusion. The viability (95% confidence interval) was 65.5 (57.5 - 73.5)%. The cell suspension was adjusted to 1 x 10^6 cells/ml and 100µl added to a cytospin slide (Shandon Cytospin 3). The slide was stained differentially (Diff Quick, Dade) and 300 cells were counted.

4.3.3 Cytokine Measurements

IL-8 was measured by a specific radioimmunoassay as detailed in Chapter 3. In short, samples were diluted appropriately in phosphate buffer and 100µl added to duplicate tubes. 25µl of rabbit anti-human IL-8 was added and incubated overnight at 4°C. A further 25µl of ^{125}I labelled human IL-8 at 15000cpm was added to all tubes and incubated overnight at 4°C. 200µl of precipitating antibody solution (see "Appendix A p178") was then added to each tube with a further incubation for 1 hour at 4°. The tubes were then spun at 3950g (Jouan, CR422) for 20 minutes at 4°C and after the supernatants had been discarded the pellets were counted in a Canberra-Packard

gamma counter. An eight point standard curve (range 5µg/L - 50ng/L) was included as were three quality controls and a background level.

TNF-α was measured by a commercially available high sensitivity ELISA (R & D Systems, Massachusetts). The assay has an intra-assay specificity <9% and an inter-assay specificity of <10.5%. The minimum detectable dose in serum is <180 fg/ml making this assay very sensitive and TNF-α can be detected in very small samples.

4.3.4 Bacteriology Sample

Endotracheal secretions are sent for routine analysis to the microbiology laboratories where they are cultured for common organisms such as group B streptococci, Staphylococcus and Coliforms. None of the infants in this study were colonised by any organism in the first 72 hours of life.

4.4 Results

4.4.1 Clinical Details

26 infants were enrolled in the study over a period of 11 months and the clinical details for all patients enrolled are given in *Table 4-1.*

Table 4-1: Clinical Details of All Infants Enrolled on the Study

	Total	Developed Chronic Lung Disease †	Did not develop CLD
Male	10	7	3
Female	16	8	8
Mean Gestation (wks) (95% CI)	28 (27, 29)	26 (24, 27)	29.2 * (28.9, 29.5)
Mean birthweight (g) (95% CI)	1102 (956, 1248)	869 (719, 1019)	1420 ** (1298, 1542)
Prenatal steroids	18	10	8
Surfactant	16	11	5

All infants survived

† Defined as oxygen requirement at 28 days of age

** $p < 0.001$ Unpaired T-test ** $p < 0.0001$ Unpaired T-test*

Abbreviations: CI = confidence intervals; CLD = chronic lung disease.

It was not always possible to get a sample from an infant at a specific time for clinical reasons, however every effort was made to get as complete a set of samples as possible.

All samples were tested firstly for interleukin-8 and only if enough sample were available was a test for TNF-α done. This means that there are fewer TNF-α results. As the cytokine data was highly skewed the values shown are medians and non-parametric statistical analysis were performed.

4.4.2 Cytokine Results for All Infants

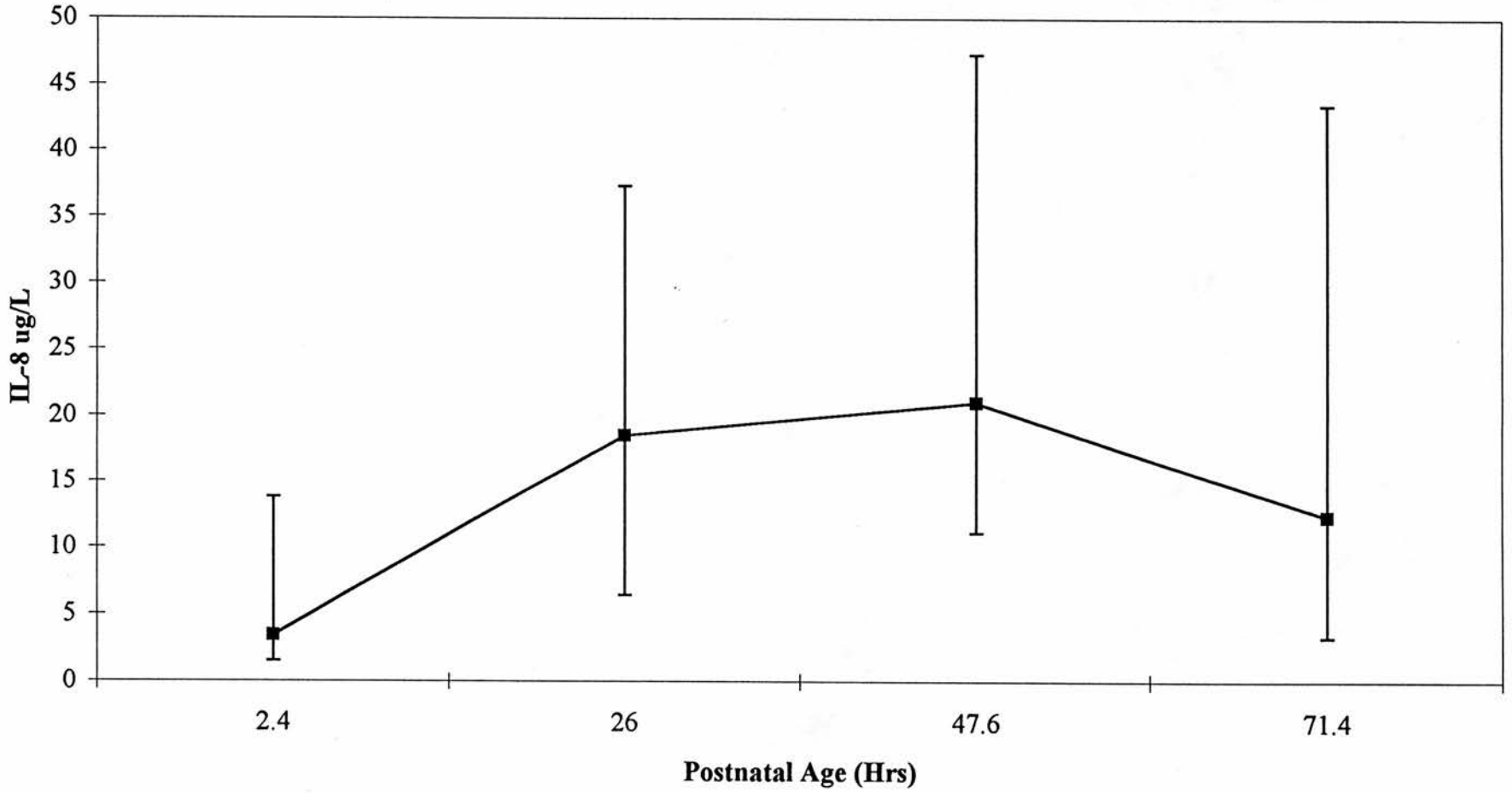
Table 4-2: TNF-α μg/L for All Infants Enrolled on Study

Time (Hrs)			TNF-α (μg/L)
Mean	**n**	**sem (Range)**	**Median (IQR)**
2.4	9	0.6 (0 - 7.9)	0
26.0	17	5.8 (14.8 - 39.6)	0 (0 - 48.6)
47.6	13	2.3 (40.6 - 59.9)	0 (0 - 54.9)
71.4	11	19.1 (63.3 - 79.9)	0 (0 - 85.3)

sem = standard error of the mean; IQR = interquartile range.

The IL-8 rises over the first 24 hours and levels over the remaining 72 hours staying relatively high in all infants. The TNF-α was undetectable in most samples so the median values are all 0. There are also too few TNF-α results on which to perform statistical analysis. Graph 4.1 is a plot of the IL-8 values for all infants (Table 10-6 Appendix B shows the raw data).

Graph 4.1: IL-8 ug/L for All Infants



Graph 4.1: IL-8 for all infants enrolled on the study. Values are medians and the interquartile range.

4.4.3 Cytokine Results Chronic Lung Disease vs. No Chronic Lung Disease

Table 4-3: TNF-α μg/L

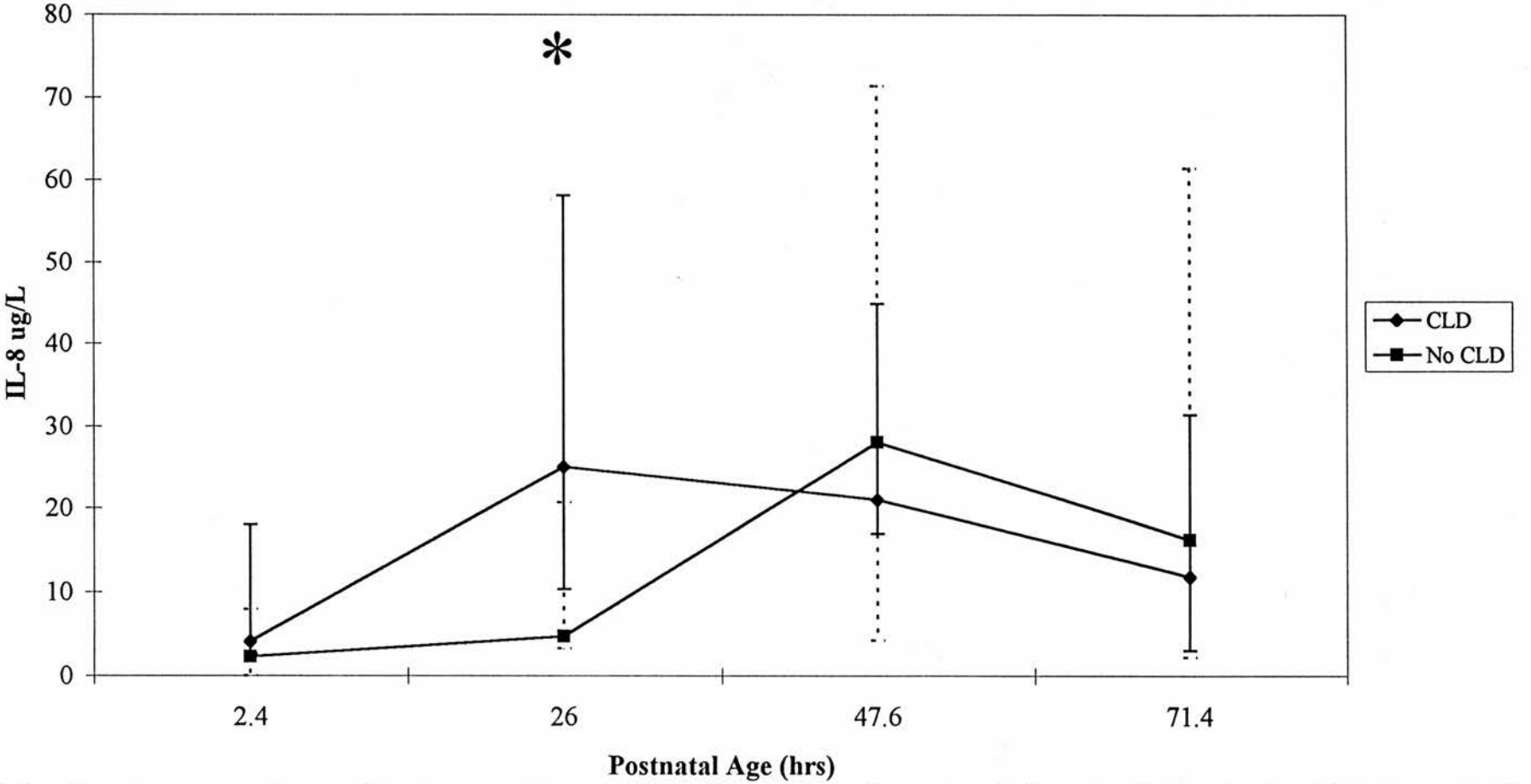
Infants divided retrospectively for those that developed CLD and those that did not.

	Chronic Lung Disease		No Chronic Lung Disease	
Time (Hrs)	**n**	**Median (IQR)**	**n**	**Median (IQR)**
2.4	5	0 0	4	0 0
26.0	12	0 (0 - 708.3)	5	0 0
47.6	9	0 (0 - 54.9)	4	0 (0 - 148.4)
71.4	7	0 (0 - 27.3)	4	42.7 (0 - 134.1)

Although the medians are all 0, TNF-α is initially only present in those infants that later develop chronic lung disease. It is undetectable in the first samples of those infants who did not develop chronic lung disease, but becomes so after 48 hours. There number of samples available are too small for statistical analysis of the TNF-α results.

IL-8 values are plotted in Graph 4.2 and raw data is in Table 10-7 Appendix B. IL-8 starts low in both groups and rises over the first 24 hours. In the infants that later develop chronic lung disease the concentration of IL-8 at 24 hours is significantly different (* = $p < 0.01$; Mann-Whitney U Test) from the group of infants that do not develop chronic lung disease. IL-8 values remain relatively high over the first 72 hours of life in both groups.

Graph 4.2: IL-8 ug/L

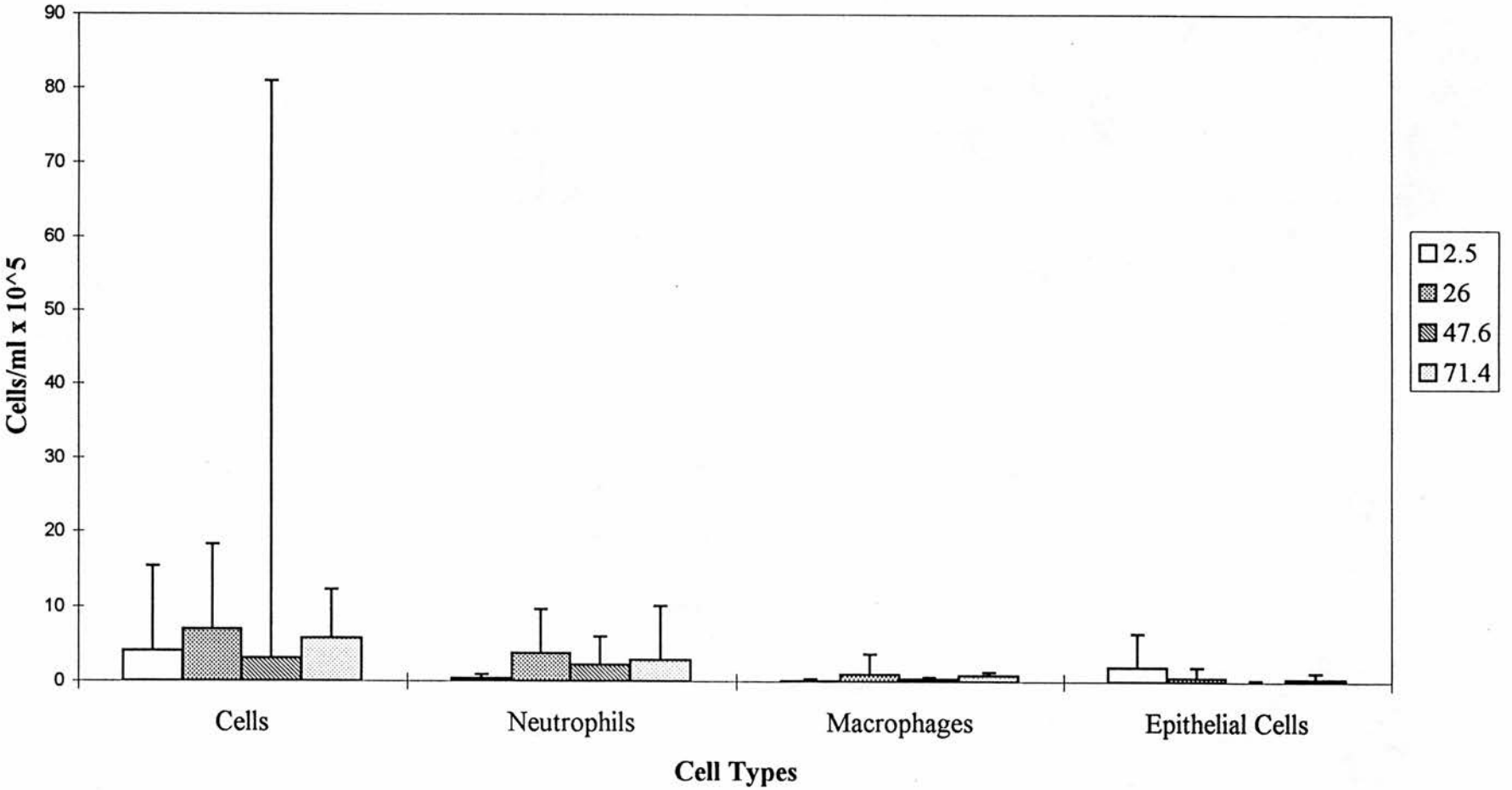


*Graph 4.2: IL-8 ug/L; values are medians and the interquartile range. Dotted error bars refer to those infants that did not develop CLD. * = $p < 0.05$ Mann-Whitney U.*

4.4.4 Cells

For all infants neutrophil numbers rise over the first 24 hours and stay high for the first 72 hours. The macrophages however never rise above 20% in the first 72 hours remaining constant for this time period. The epithelial cells constitute 95% of the initial samples but fall rapidly over the first 24 hours remaining at about 6% at 72 hours.

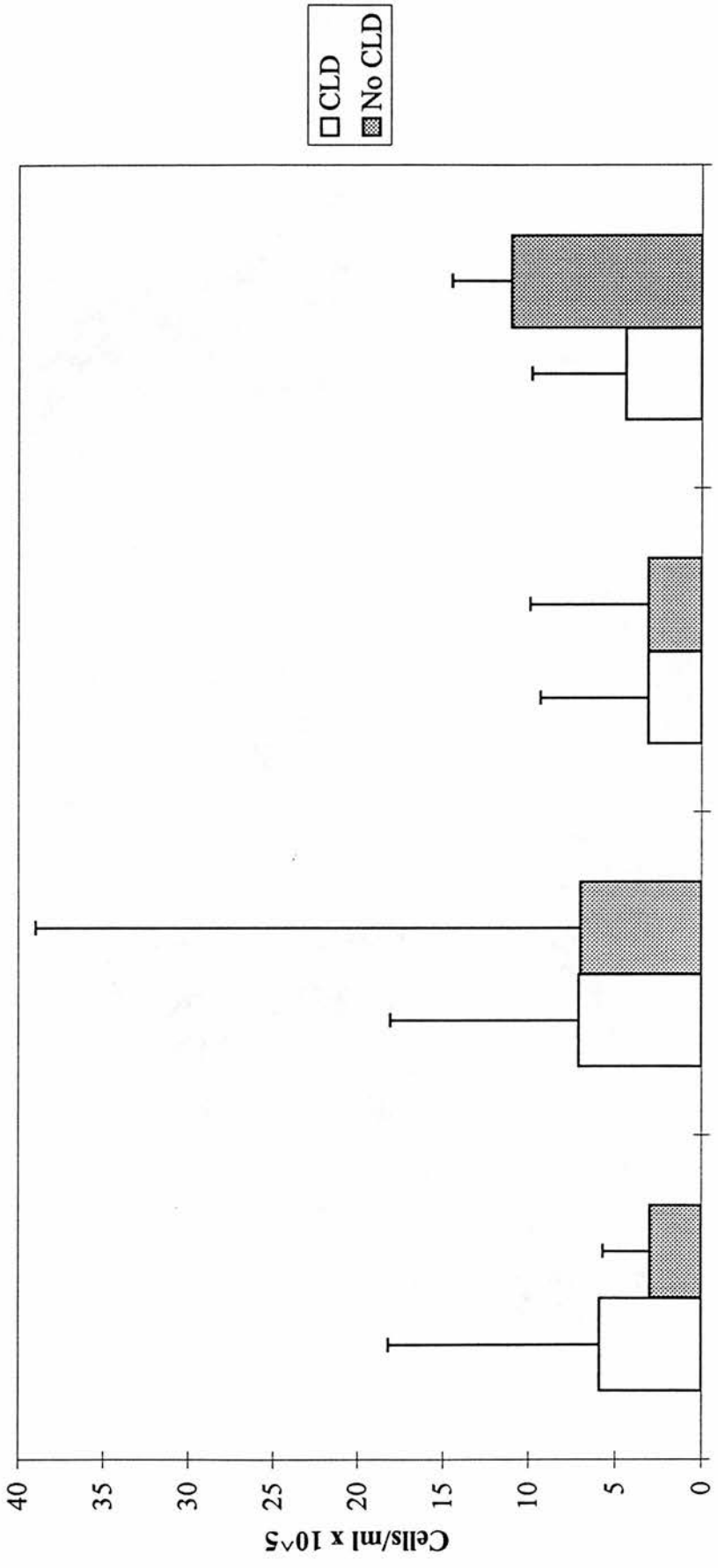
Graph 4.3: All Infants Median Total Cell Counts



Graph 4.3: Median cells/ml (x 10^5) for all infants enrolled on the study. Error bars are 75th quartile of the interquartile range.

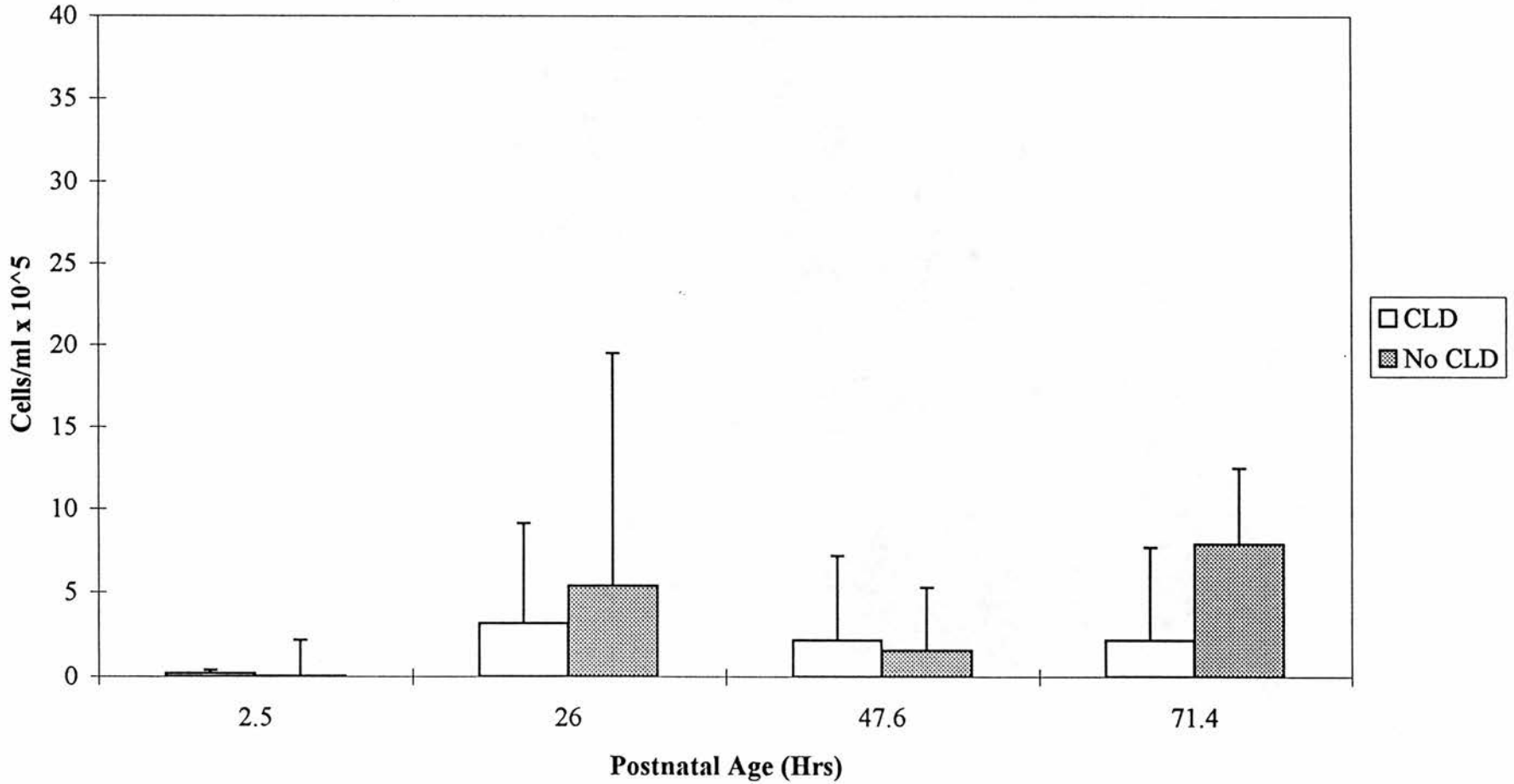
4.4.5 Cell Results Chronic Lung Disease vs. No Chronic Lung Disease

When the data is divided retrospectively into two groups, those infants that developed CLD and those that did not, the patterns between the two groups remains essentially the same. The only significant difference is between the first sample of epithelial cells. The Graphs 4.4 to 4.7 display these results.



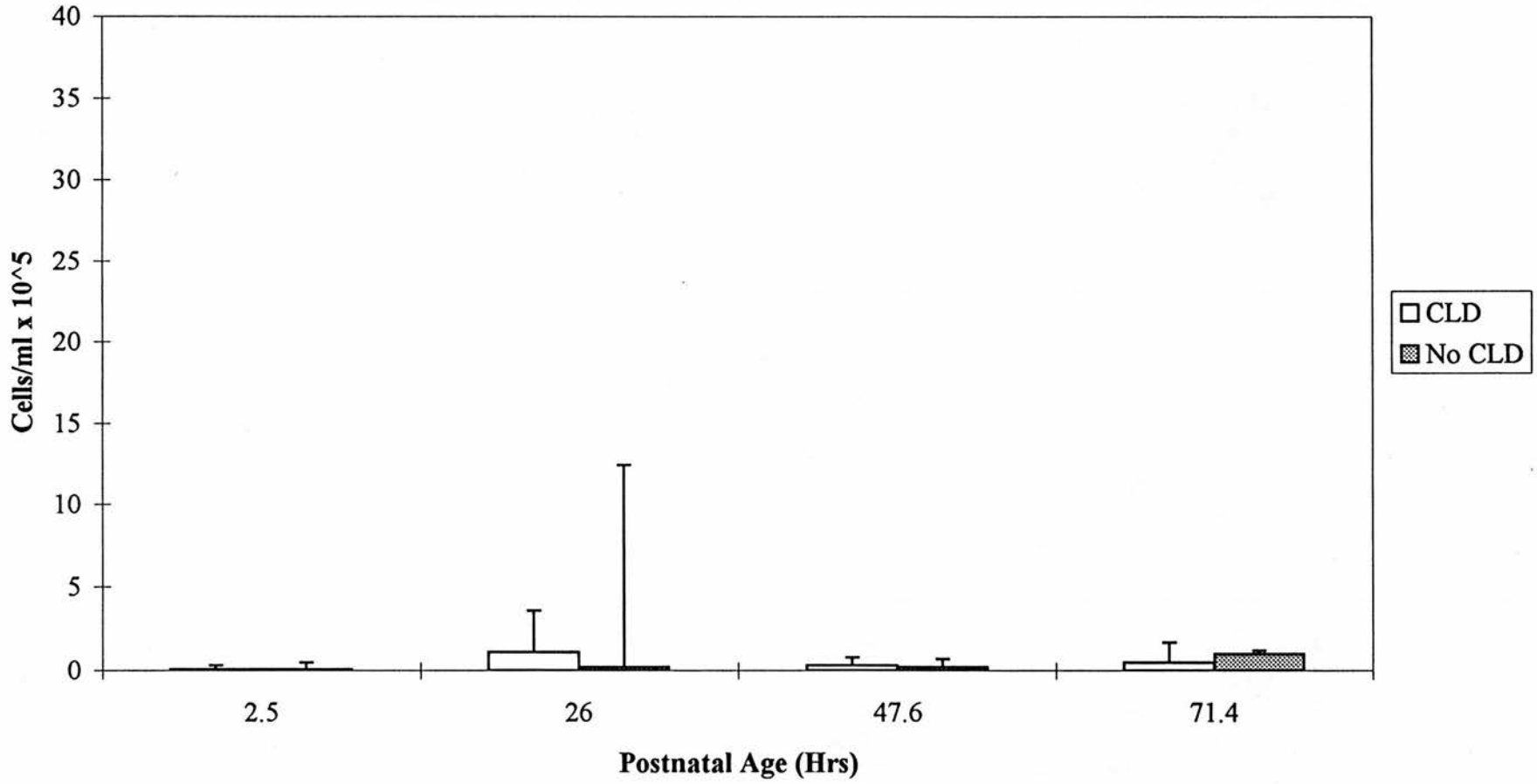
Graph 4.4: Median cell counts (x 10^5). Error bars are 75th percentile of the interquartile range.

Graph 4.5: Neutrophil Count



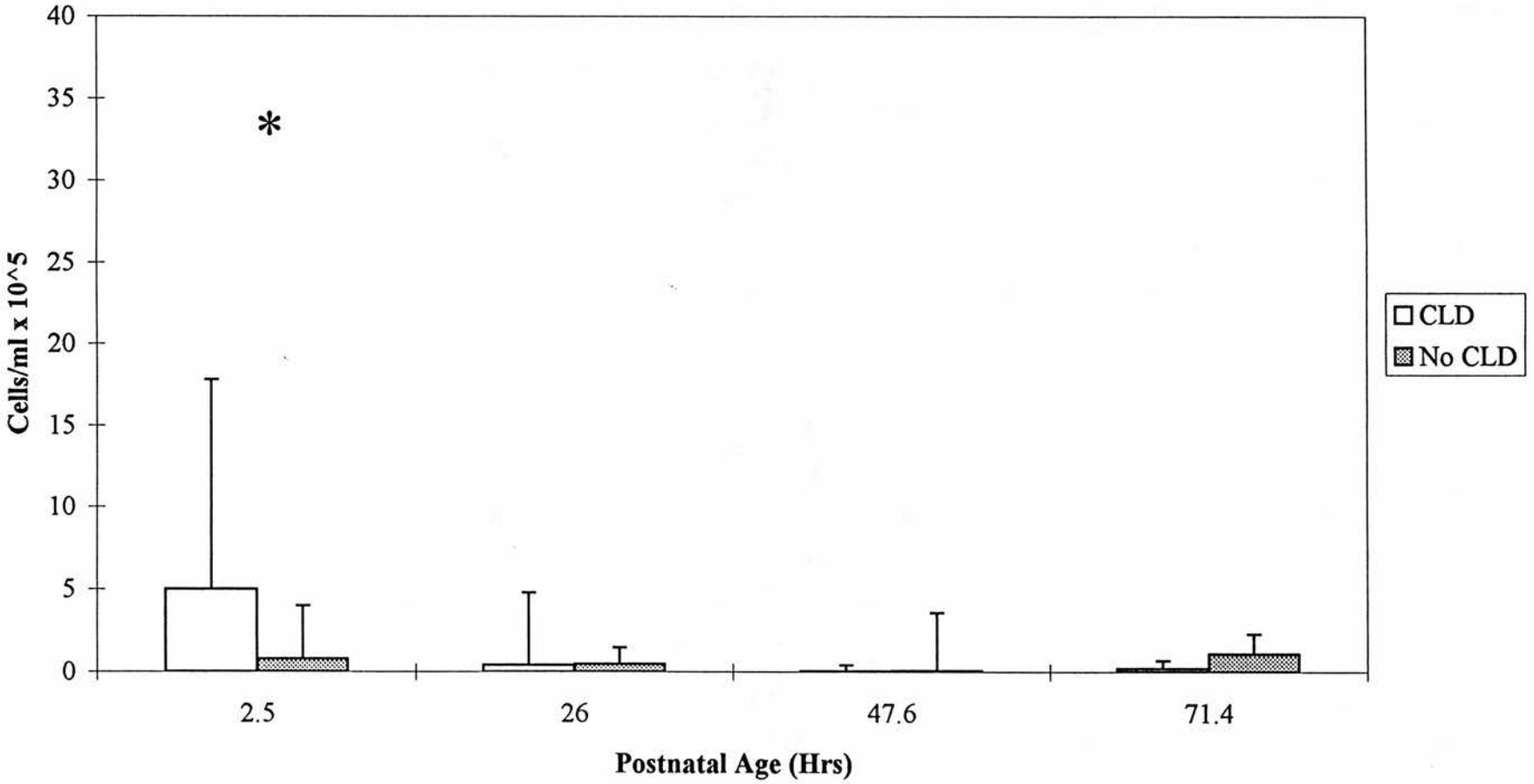
Graph 4.5: Median neutrophil counts (x 10^5). Error bars are the 75th percentile of the interquartile range.

Graph 4.6: Macrophage Count



Graph 4.6: Median macrophage counts (x10^5). Error bars are the 75th percentile of the interquartile range.

Graph 4.7: Epithelial Cell Count



*Graph 4.7: Median epithelial cell counts (x10^5). Error bars are the 75th percentile of the interquartile range. * = $p<0.05$ Mann Whitney U.*

4.5 Discussion

As expected the group that developed chronic lung disease had a significantly lower birthweight and gestation, but showed no other statistically significant differences for other clinical characteristics.

TNF-α was only detectable over the first 24 hours in the infants that later developed chronic lung disease. There were, however, too few TNF-α samples to show if this was a significant difference. The initially high level of TNF-α in this group does suggest an overall increase in inflammatory response and fits with the significantly increased level of IL-8 seen at 24 hours in the infants that developed chronic lung disease. We know that TNF-α alone, as one of the pro-inflammatory cytokines, is enough to increase the secretion of IL-8 from lung fibroblasts (Smart *et al.*,1994) (own data chapter 7).

The inflammatory cells present in the bronchoalveolar lavage samples were as expected from other studies. Over the first 72 hours, an initially high % of epithelial cells was replaced by neutrophils which remained high in both groups. The level of macrophages remained constant not going above 15% of the total cell population.

The infants that later developed chronic lung disease had a higher initial level of epithelial cells which may reflect that the ventilator damage these infants received initially was greater than in infants that did not develop chronic lung disease.

5. Genital Mycoplasmas and Erythromycin

5.1 Introduction

Recent studies have suggested an association between genital mycoplasma infection in the lungs of preterm infants and the development of chronic lung disease, but these organisms have not been shown to be causal. Their mode of action is unknown but given that infants that develop chronic lung disease have a persistent inflammatory response in their lungs, these organisms may be responsible for it.

We wished to test whether treatment with erythromycin would reduce the problem of chronic lung disease in infected infants.

We were also interested in the possible anti-inflammatory properties erythromycin, which may prove significant in this group of infants.

5.2 Study Design

This was a randomised study but it was not double blind. There were three people involved, a consultant neonatologist, a research nurse and myself. Either the consultant or myself were on call through the night (between 5 pm and 8 am) and were immediately alerted to any new infants admitted to the unit. Permission was obtained from the parents to enrol the infant in the study and they were then randomised to receive erythromycin or not. I was not aware which infants were in the treatment group. For the next four days a research nurse collected and processed the samples which were stored until analysed by me.

5.2.1 Patient Criteria

Any infant admitted to the Neonatal Intensive Care Unit, ≤ 30 weeks gestation who was immediately ventilated. Exclusions were those infants with any congenital abnormalities. Infants were randomised to receive 15 mg/kg/dose of erythromycin which was infused intravenously three times a day until the baby was tolerating feeds,

when treatment was given orally. There was no placebo for those in the non-treatment group. All other treatments were as per normal unit policy.

5.2.2 Definition of Chronic Lung Disease

Oxygen requirement with abnormalities on x-ray at 36 weeks corrected gestational age which predicts abnormal findings at follow-up in infants ≤ 30 weeks gestation (Shennan *et al.*,1988).

5.2.3 Measurement of the Severity of Lung Disease

Assessing the severity of lung disease can be difficult, but is important in predicting the likelihood of an infant of further hypoxemic episodes and readmission to hospital. Measuring the mean individual alveolar/arterial oxygen saturation ($AaDO_2$) (Iles *et al.*,1996a) of these infants may give some indication of the severity of chronic lung disease, and we wished to see if infected infants had more severely damaged lungs compared to uninfected infants. $AaDO_2$ was measured at 36 weeks gestational age from an arterial blood gas done while the baby was in a steady state breathing 50% oxygen in a headbox.

5.3 Methods

5.3.1 Sample Collection and Processing

Once enrolled in the study, two bronchoalveolar lavage samples (see "Chapter 2 p30") were taken. The first as soon as possible after admission, and the second at approximately 4 hours postnatal age. Both were kept at 4°C until processed. The first sample was taken for bacterial culture and PCR and the second for cell staining and cytokine measurement. Mean time until the second sample was processed was 1 hour and 37 minutes ± 10 minutes (95% confidence limits). If surfactant treatment was clinically indicated then the second bronchoalveolar lavage was taken before surfactant administration. Samples were then taken daily whilst the infant remained ventilated, up to the first 5 days of life.

Samples were prepared as described previously (see "Chapter 2 p30"), and if any RPMI-1640 was added the volume was noted. The mean sample recovery volume was 90µl ± 15µl (95% confidence limit) and the range was 10 to 500µl. Five samples were >500µl and this was because after the sample was taken the nurse flushed the line with 1ml saline. These samples were used but not diluted as the others were and all dilutions were noted and accounted for in the final analysis.

5.3.2 Culture of the Genital Mycoplasmas

Previous studies have relied on culturing the genital mycoplasmas, but these organisms are difficult to culture due to pH sensitivity. It is more sensitive to use polymerase chain reaction (PCR) amplification of the urease genes of *U. urealyticum,* which also results in a more rapid test, taking < 24 hours compared to 2 - 5 days for culture (Blanchard *et al.*,1993). The samples were examined for *U. urealyticum* using both culture and PCR. We do not have suitable PCR primers for *M. hominis* and so results for these organisms are given for culture only.

Culture was carried out by Mr S. Fergusson, Medical Microbiology, Edinburgh University. Samples were inoculated into 1.8 mls ureaplasma broth or mycoplasma broth and serial 1:10 dilutions were made over 5 pots. These were incubated at 37°C for at least 72 hours and checked twice daily. Any pots that had turned red were deemed to be positive for growth. The colour change is due to an increase in the pH and the lowest concentration which turned red was taken as the titre.

5.3.3 Ureaplasma PCR

This was carried out by Dr. N. Cunliffe, Medical Microbiology, Edinburgh University as previously described (Cunliffe *et al.*,1996).

All of the culture positive samples were PCR positive, but 6 of the 100 samples tested were culture negative and PCR positive, giving a 93% agreement between methods. The results given in this chapter for the *Ureaplasma* are the PCR positives and for *M. hominis* the culture positives.

5.3.4 Urea Measurements

A 50µl aliquot of the bronchoalveolar lavage sample (when available) was heated for 1 hour at 60°C in a water bath to kill any organisms and then sent to Paediatric Biochemistry, RHSC for urea measurement. The Hitachi Kinetic UV method is used routinely in this laboratory and the principle of the assay is detailed in Figure 5.1.

$$\text{urea} + H_2O \xrightarrow{\textit{urease}} 2\,NH_3 + CO_2$$

$$2\,\alpha\text{-oxoglutarate} + 2\,NH_4^+ + 2\,\text{NADH} \xrightarrow{\textit{glutamate dehydrogenase}} 2\,\text{L-glutamate} + 2\,NAD^+ + 2\,H_2O$$

Figure 5-1: Hitachi Kinetic UV method for measuring urea relies on the production of NAD^+ which is directly proportional to the amount of urea (mmol/L).

The rate at which NAD^+ is produced is directly proportional to the urea concentration and is measured by the decrease in absorbance at 340nm by a BCL Hitachi analyser. The method relies on a two point calibration and is linear up to 50 mmol/L urea with a lower detection limit of 0.3 mmol/L. The normal blood reference range in infants is 1.4 - 5.4 mmol/L.

Urea measurements were also available from serum samples taken daily and routinely in our unit. To correct for dilution of bronchoalveolar lavage samples the following equation is used;

$$\underline{\text{BAL urea}} = \text{Serum urea} \times \text{BAL volume}$$

where BAL stands for bronchoalveolar lavage.

5.3.5 Arterial Oxygen Measurements

Measurements were taken before discharge from the unit on all infants that survived. This was carried out by Dr. Richard Iles and detailed methodology are described elsewhere (Iles *et al.*,1996a).

5.3.6 White Blood Cell Counts

On admission to the unit blood samples are taken and the white cell counts measured.

5.3.7 Pathology of Placenta

The placentas were all sent for routine pathology, where if an influx of immune cells was confirmed by histological examination, chorioamnionitis was diagnosed.

5.3.8 Cytokine Measurements

Interleukin-8

In-house radioimmunoassay as detailed in Chapter 3. 50µl samples were diluted 1:6 and 1:36 in RPMI-1640 (PFC, Edinburgh) and measured in duplicates.

Tumour Necrosis Factor-α

High sensitivity kit (R&D Systems, Cat. No. HSTAO) with standard curve range of 0.5 - 32 pg/ml and a sensitivity of <180 fg/ml.

Interleukin-1 β

High sensitivity kit (R&D Systems, Cat. No. HSLBO) with standard curve range of 0.125 - 8 pg/ml and a sensitivity of 100 fg/ml

Samples for both the TNF-α and IL-1β were diluted 1:20 in RPMI-1640 to obtain enough volume to test in duplicates. Results were expressed as an average of these two values extrapolated from a standard curve.

5.3.9 Cells

The cells were prepared as previously described (see "Chapter 2 p30"). One slide was stained differentially using Diff-Quik (Dade) and any others were air dried overnight before storage at -20°C with a silicon packet to act as a desiccant. These slides were then stained immunohistochemically (see "Section 6.5 p124").

5.4 Results

5.4.1 Statistical Analysis of Data

The distributions of data in all sets was highly skewed so the data was analysed after log transformation. Measurements below the limit of detectability were assigned an arbitrary value of 10 before transformation. The data was split initially as a pre-surfactant and post-surfactant sample and thereafter daily up to 5 days. If an infant had more than one measurement in any time period then the values were averaged after log transformation. Mann-Whitney U tests were performed on numerical data and Chi square tests were performed on binary data.

5.4.2 White Cell Counts

Of the 76 infants enrolled white cell counts within the first 24 hours were taken for 67 infants. Mean (95% confidence interval) white cell count for the infected babies was 7.26 (1.27) and for the uninfected babies was 9.93 (0.52). This difference was not significant (p=0.897) using a Mann-Whitney U test.

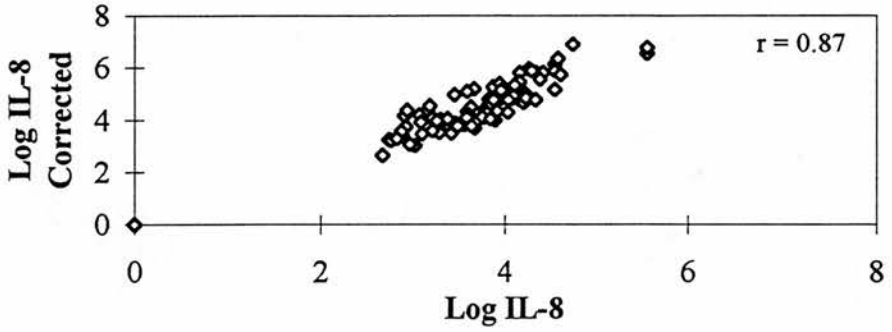
5.4.3 Urea

Urea measurements were made when enough sample was available. However, of 264 bronchoalveolar lavage samples taken in the study there was enough sample only to test 145 for urea. Of these 100 (69%) were below the limit of detection of the urea assay. Because the majority of samples could not be measured for urea the results presented in this chapter are not corrected for with urea.

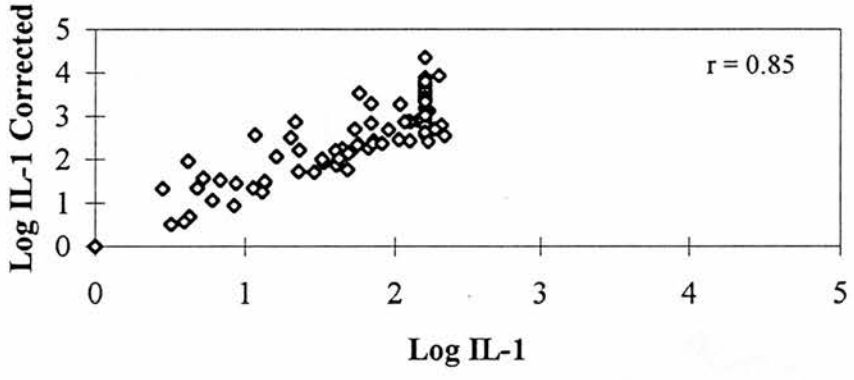
The samples which could be corrected with urea were compared in a separate analysis. Graphs 5.1 to 5.3 are scatter plots for the three cytokines, TNF-α, IL-8 and

IL-1β, with log of the uncorrected cytokine on the x-axis and log of the urea corrected cytokine on the y-axis. The correlation coefficients are shown on each graph and in each case are high. The variation between different samples is much greater than the variation in the amount of correction. The rank order of the corrected values are similar to the uncorrected and the results are not likely to be greatly affected by correction with urea.

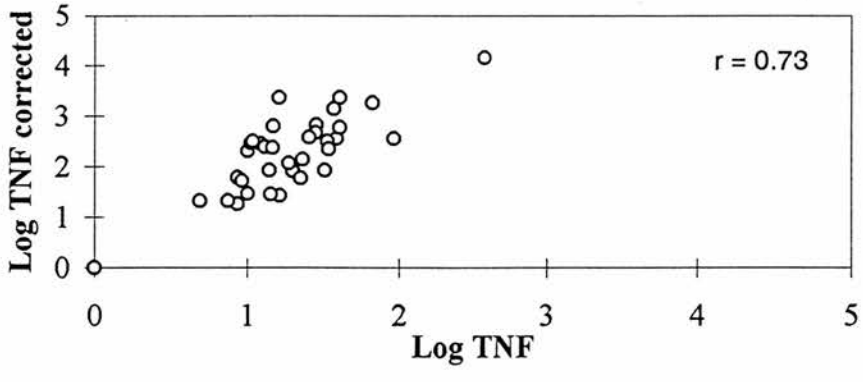
Graph 5.1: Scatter Plot of IL-8



Graph 5.2: Scatter Plot for IL-1



Graph 5.3: Scatter Plot for TNF



5.4.4 Clinical Details for All Infants

The study ran for just over 23 months during which time 172 infants ≤ 30 weeks gestation were admitted to the Neonatal Unit. Of these 52 were not ventilated, 12 died within hours of admission, 3 had congenital abnormalities, and 28 infants were missed due to there being no one available to collect the samples. Only 1 infants parents refused permission. In all 76 infants were randomised into the study but one infant was extubated within 24 hours and no samples were taken.

Table 5.1 gives the clinical details for all infants enrolled on the study.

Table 5-1: Data are Medians (range)

	All Infants
n	75
Birthweight (g)	1040 (500 - 2300)
Gestation (wks)	28 (23 - 30)
Length of Stay (days)	48 (1 - 214)
Time on Ventilator (days)	6 (1 - 80)
CLD (O_2 at 36wks)	24
Ureaplasma	9
Ante-natal steroids	18
Chorioamnionitis	20[†]
Surfactant	51
Died	13
$AaDO_2$	23.6 (2.8 - 35.2)

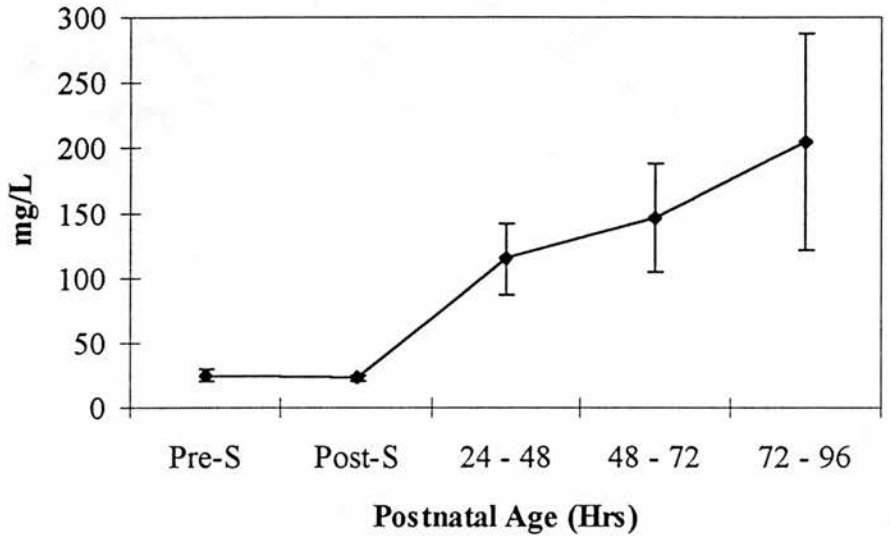
[†] 8 infants were not tested

5.4.5 Cytokine and Cell Results

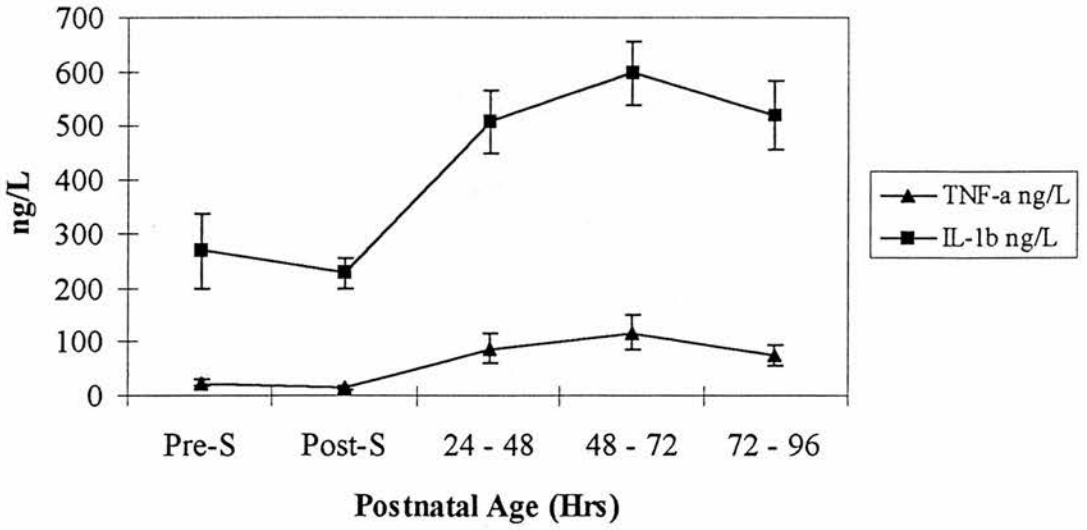
Graphs 5.4 and 5.5 are plots of the cytokine data for all infants enrolled on the study. The cell data is given in Graph 5.6.

Graphs 5.4 and 5.5: Cytokines in bronchoalveolar lavage for all infants in the study group. Data are means, error bars are 95% confidence intervals.

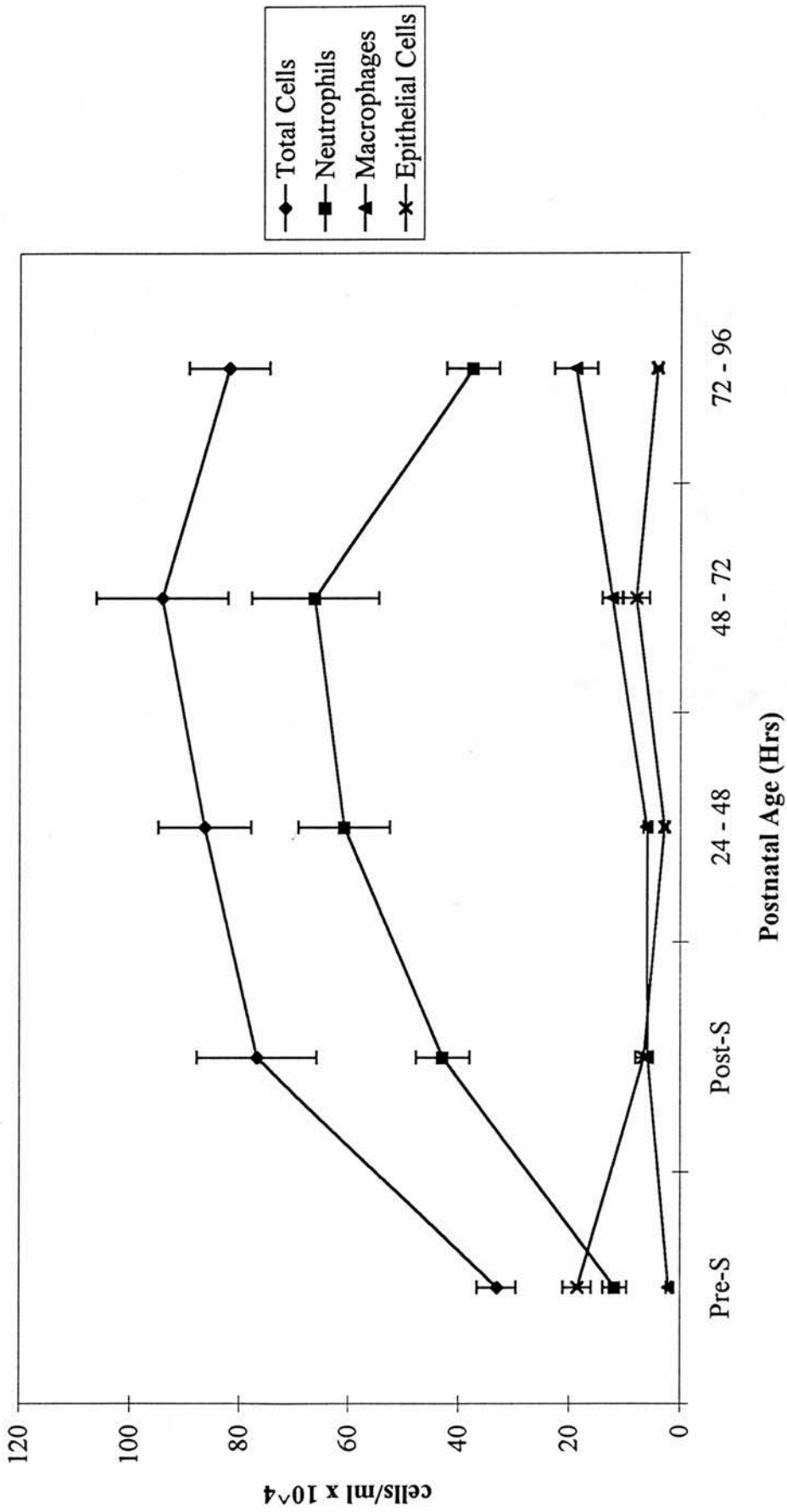
Graph 5.4: IL-8 Results



Graph 5.5: IL-1 beta and TNF-alpha Results



Graph 5.6: Cells Results



U. urealyticum was isolated in 13% of the study group which is lower than the incidence of *Ureaplasma* infection reported from other studies (Payne *et al.*,1991; Wang *et al.*,1988; Cassell *et al.*,1988), and less than half the rate of our own pilot study (Iles *et al.*,1996b).

IL-8 was low initially and rose over the first 48 hours reaching a plateau at 96 hours. The median TNF-α values for all samples was zero and TNF-α was virtually undetectable in any samples until after 24 hours. Our sampling regime which is only a small window in 24 hours may be unsuitable to detect TNF-α. IL-1β follows the same pattern as IL-8 rising over the first 24 hours and remaining at a plateau for the next 72 hours.

The total cells for all infants rises after 24 hours and then remains level for the duration of the study. The percentage of different cell types within the total cell numbers changes dramatically. Initially the epithelial cells are the predominant cell type, but they are replaced by neutrophils after 24 hours, which then remain the predominant cell type. The macrophages gradually rise over the course of 96 hours reaching a median of 16% of the total cell count of the sample at 72 - 96 hours.

The data can be further analysed by comparing the cytokine and cell data in relation to several treatments: erythromycin and surfactant, and to outcome: development of chronic lung disease, and the severity of that disease: $AaDO_2$. We were also interested in whether the group that were infected would have any different inflammatory responses to the uninfected group.

5.5 Treatment Vs Control Group

5.5.1 Results

5.5.2 Clinical Details

Table 5-2 gives the clinical details sub-divided into infants that were in the erythromycin treatment group and those that were not.

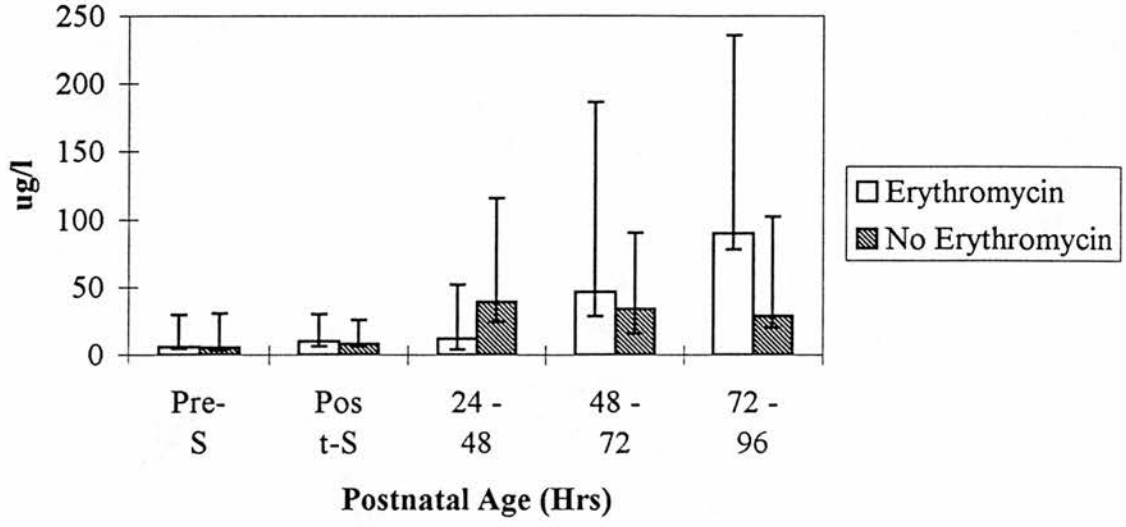
Table 5-2: Data are medians (range).

	Treatment Group	Control Group	p or *F* values
n	35	41	
Birthweight (g)	1032.5 (590 - 2300)	1050 (500 - 1670)	0.93
Gestation (wks)	28 (24 - 30)	29 (23 - 30)	0.59
Length of Stay (days)	60.5 (2 - 185)	45 (1 - 214)	0.68
Time on Ventilator (days)	6 (1 - 41)	5 (1 - 80)	0.39
CLD (O_2 at 36wks)	13	11	0.46
Males	17	30	**0.035**
Ureaplasma	3	6	0.61
Ante-natal steroids	9	9	0.64
Chorioamnionitis	9	10	1.00
Surfactant	25	26	1.00
Died	4	8	0.76
$AaDO_2$	21.8 (2.8 - 34.3)	24 (13.7 - 35.2)	0.051

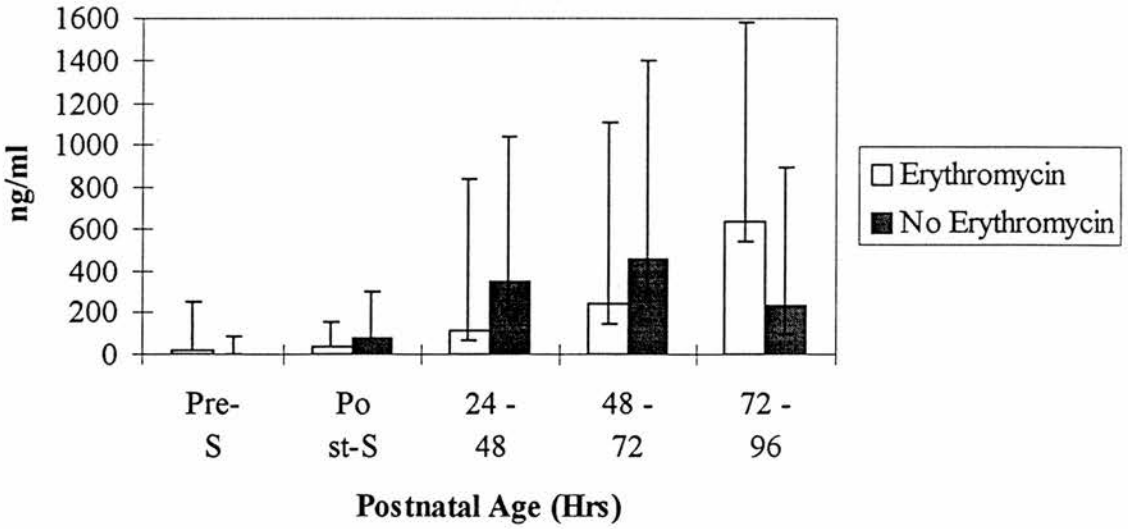
There was only one significant difference between the treatment and the control group for the details given in Table 5-2. The number of males in the control group was significantly higher than the treatment group. The incidence of chronic lung disease, defined as oxygen requirement at 36 weeks, was the same in both groups and the difference in the severity of lung disease ($AaDO_2$) did not reach statistical significance.

Graphs 5.7 - 5.8: Cytokines in bronchoalveolar lavage comparing the treatment and control groups. Data are medians and error bars are the interquartile range.

Graph 5.7: IL-8 Results

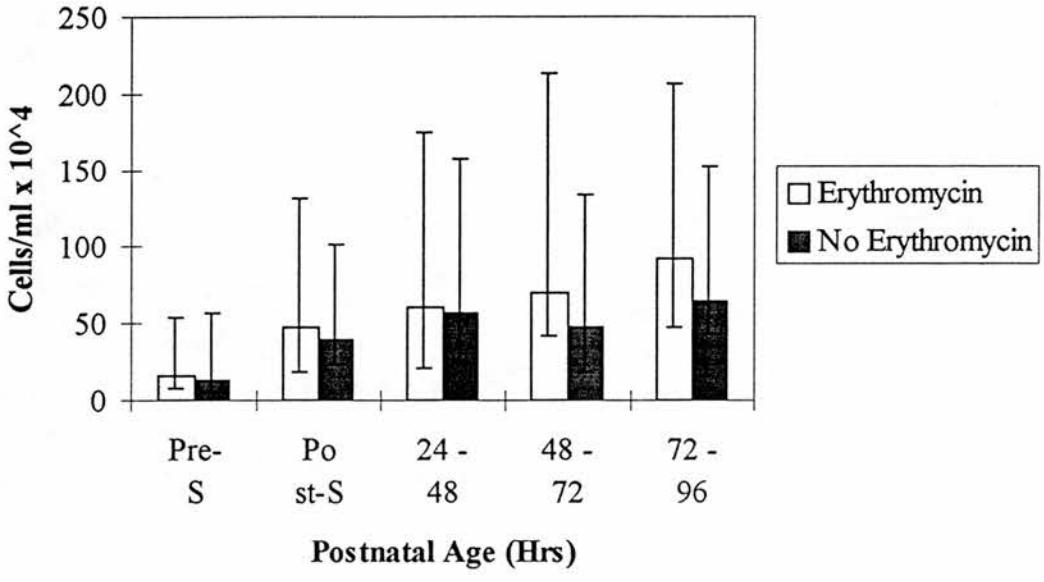


Graph 5.8: IL-1 beta Results

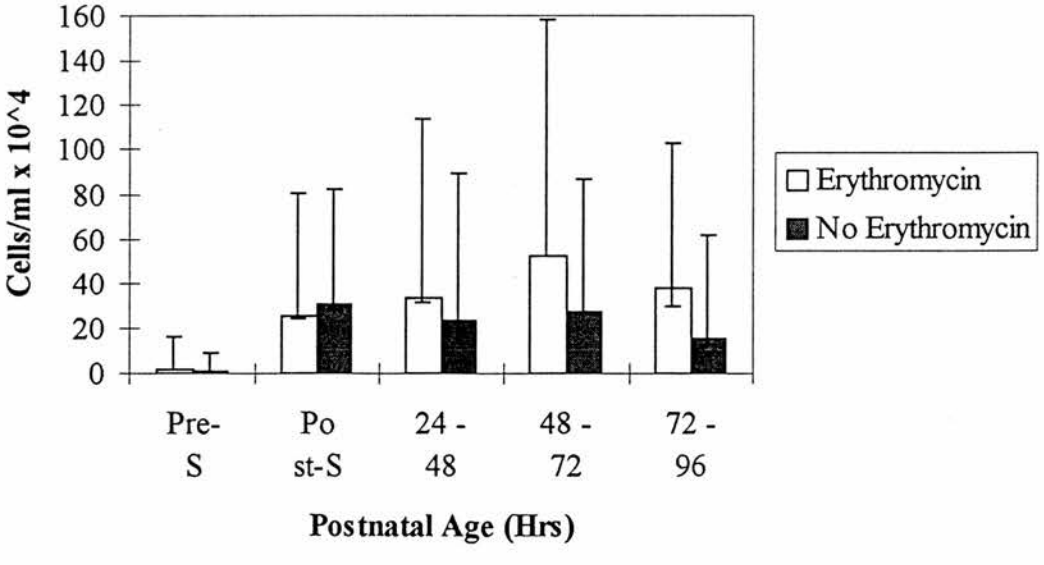


Graphs 5.9 and 5.10: Total cells and neutrophils in bronchoalveolar lavage comparing the treatment and control groups. Data are medians and error bars are the interquartile range.

Graph 5.9: Total Cells

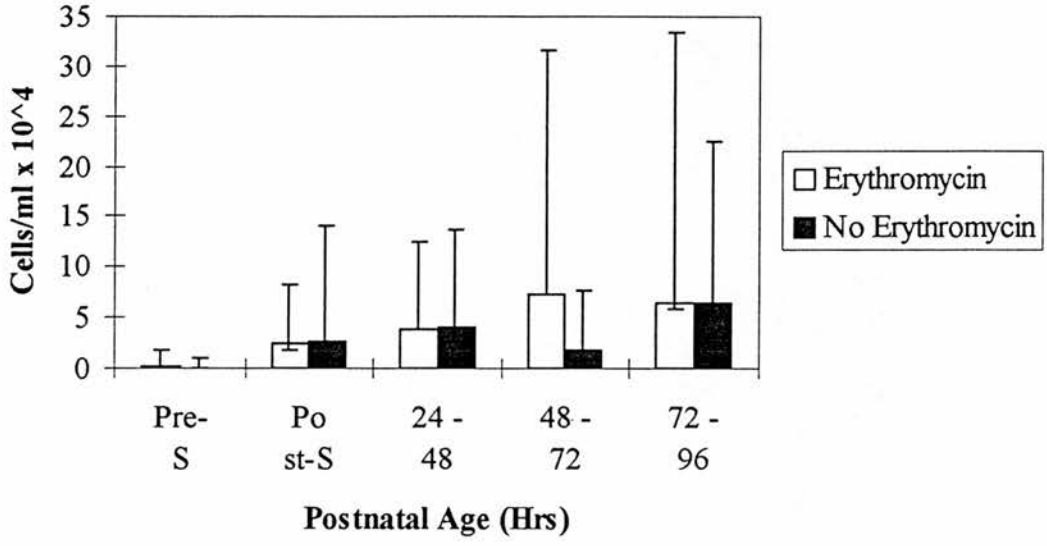


Graph 5.10: Neutrophils

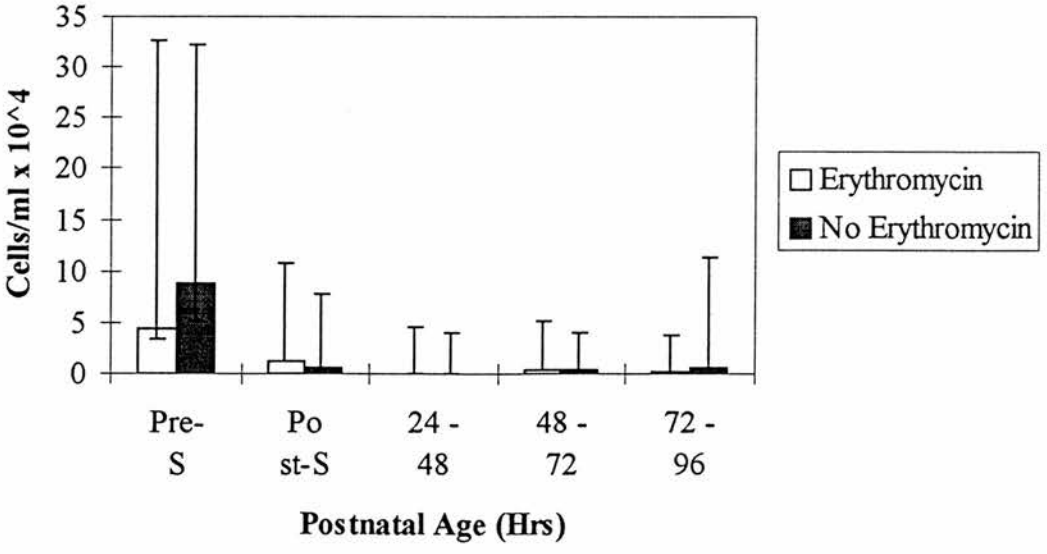


Graphs 5.11 and 5.12: Macrophages and epithelial cells in bronchoalveolar lavage comparing the treatment and control groups. Data are medians and error bars are the interquartile range.

Graph 5.11: Macrophages



Graph 5.12: Epithelial Cells



There are no significant differences between the two groups at any time point for any cytokine marker. IL-8 rises in both groups over the study period. IL-1β starts higher in the non-treated group before any interventions but after 24 hours both groups follow a very similar pattern. TNF-α is initially only detected in the treatment group both immediately before surfactant (and treatment) and after surfactant. However the differences are not significant. As all median values for TNF-α are 0 this data is not graphed.

The total cells for both groups rise over the first 24 hours and remain at a constant high level thereafter. The epithelial cells drop drastically over the initial 48 hours and remain relatively low. There are no significant differences between the two groups. The macrophages and neutrophils rise sharply after 24 hours with the neutrophils always being the predominant cell type. In the treatment group both macrophages and neutrophils rise at about 48 hours and for both cell types the difference at 72 hours is significant at $p < 0.05$ (Mann - Whitney U).

5.6 Ureaplasma Infection Vs No Ureaplasma Infection

5.6.1 Introduction

Although the infection rate was lower than expected the data allows us to analyse the effect colonisation had on the inflammatory response, outcome and severity of lung disease.

5.6.2 Results

5.6.3 Clinical Details

Table 5.3 gives the clinical details for infants divided into the group that were genital mycoplasma positive and those that were negative.

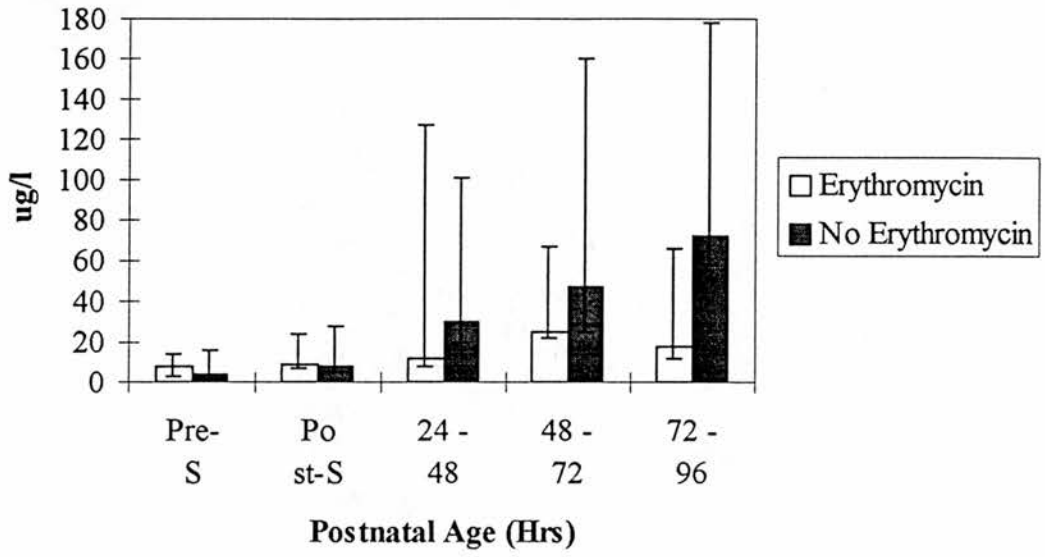
Table 5-3: Data are medians (range).

	Infected	Not Infected	p or *F* values
n	9	51	
Birthweight (g)	880 (765- 1450)	1025 (500 - 1820)	0.12
Gestation (wks)	27 (24 - 29)	28 (23 - 30)	0.26
Length of Stay (days)	81 (7 - 122)	50 (1 - 214)	0.12
Time on Ventilator (days)	14 (2 - 80)	6 (1 - 63)	0.14
CLD (O_2 at 36wks)	2	20	0.46
Ante-natal steroids	4	26	1.00
Chorioamnionitis	4	11	0.20
Surfactant	4	40	**0.048**
Died	3	10	1.00
$AaDO_2$	25.2 (14.6 - 31.9)	23.7 (8.4 - 35.2)	0.47

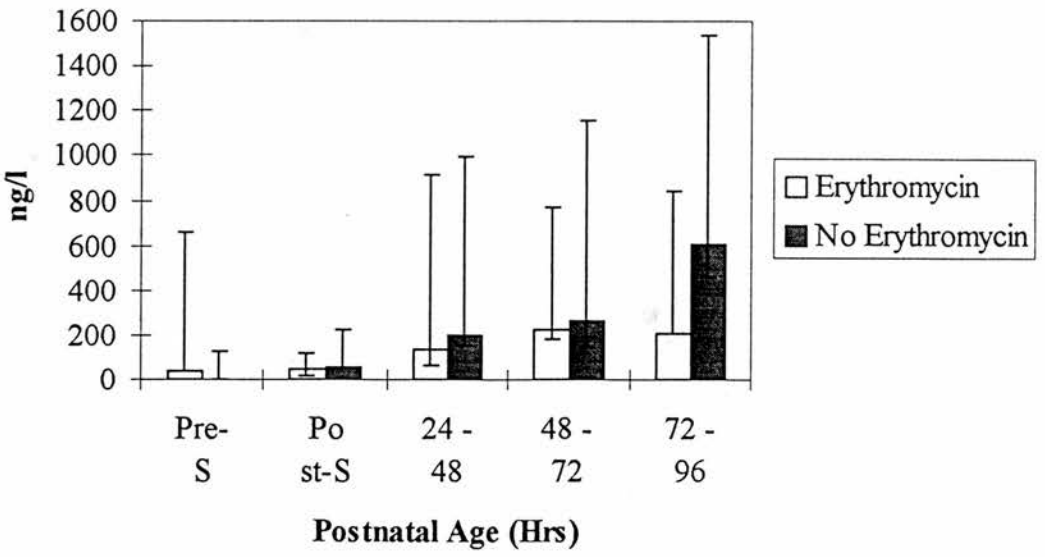
There was only one significant difference at $p < 0.05$; the infected infants were less likely to receive surfactant. There were no other significant differences between the two groups for the rest of the characteristics outlined in Table 5.10. There was a trend towards infected infants being smaller and staying in the unit longer, but there was no difference in the severity of chronic lung disease ($AaDO_2$) in the infected group.

Graphs 5.13 - 5.14: Cytokines in bronchoalveolar lavage comparing infants that were infected with mycoplasmas (I) with those that were not (NI). Data are medians and error bars are the interquartile range.

Graph 5.13: IL-8 Results

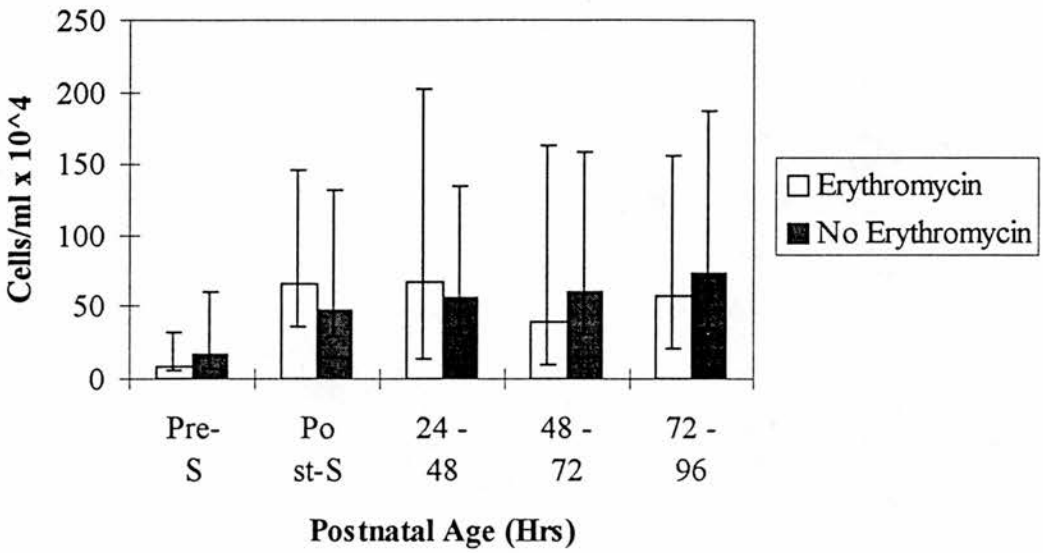


Graph 5.14: IL-1 Results

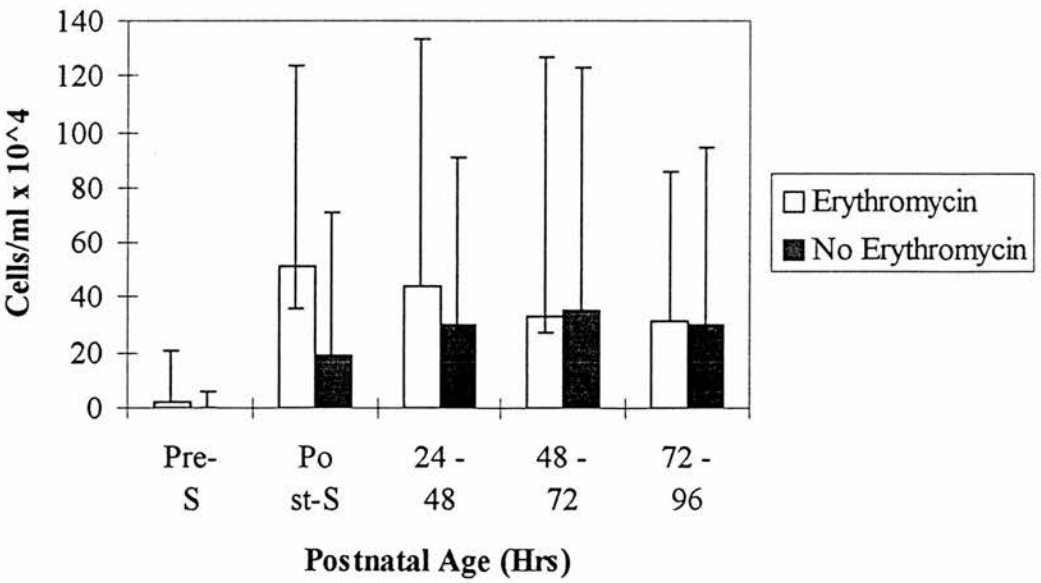


Graphs 5.15 and 5.16: Total cells and neutrophils in bronchoalveolar lavage comparing infants that were infected with mycoplasmas (I) with those that were not (NI). Data are medians and error bars are the interquartile range.

Graph 5.15: Total Cells

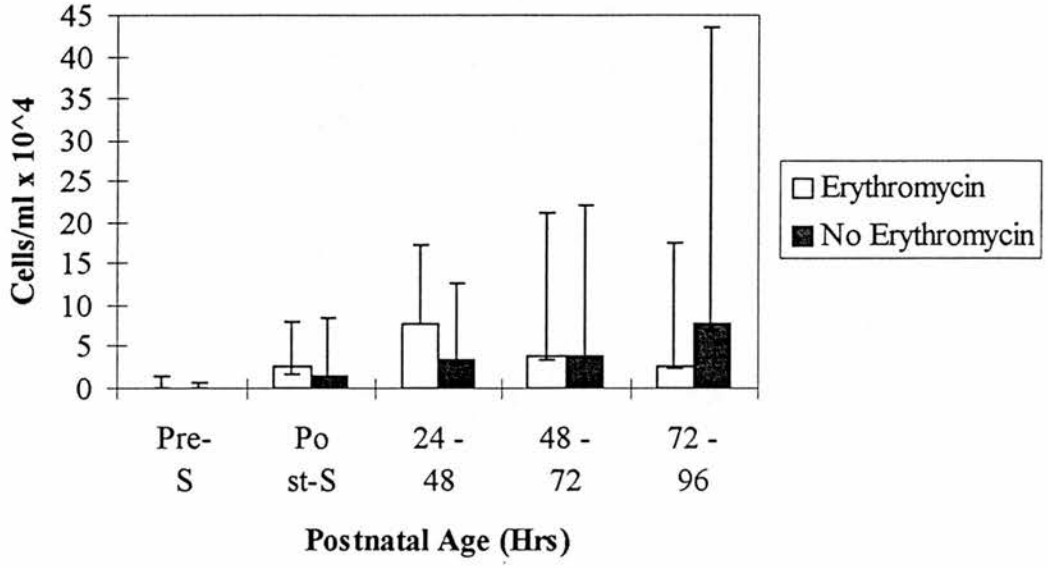


Graph 5.16: Neutrophils

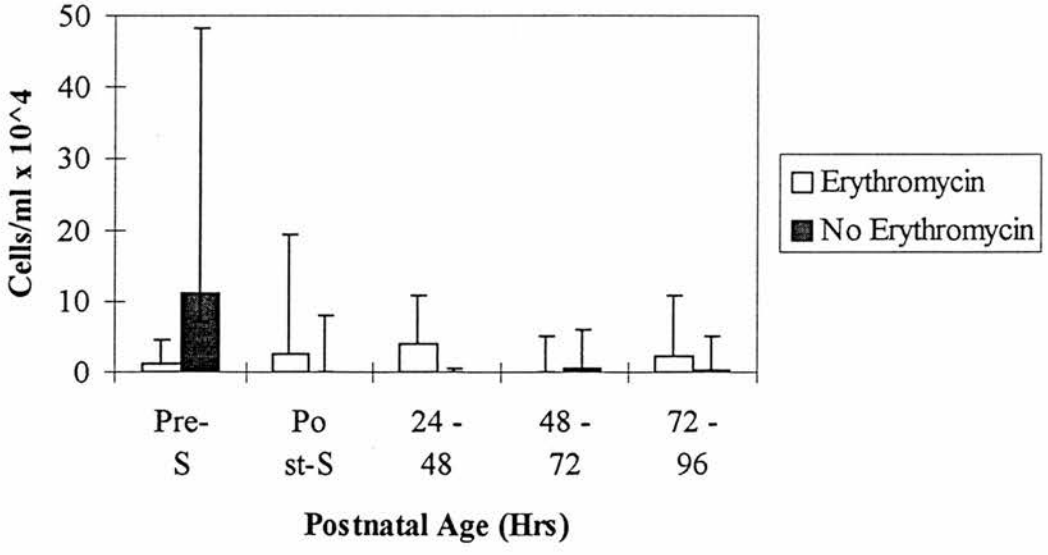


Graphs 5.17 and 5.18: Macrophages and epithelial cells in bronchoalveolar lavage comparing infants that were infected with mycoplasmas (I) with those that were not (NI). Data are medians and error bars are the interquartile range.

Graph 5.17: Macrophages



Graph 5.18: Epithelial Cells



Both IL-1β and IL-8 start higher in the infected group but the differences are not significant. They both rise over the study period and remain elevated at 96 hours. IL-8 is significantly higher in the non-infected group at 48 - 72 hours ($p < 0.05$). TNF-α is undetectable in most samples but is significantly higher in the infected group at 24 hours ($p < 0.01$). The TNF-α data is not graphed.

For the cell data the only significant difference at $p < 0.01$ is in the very first sample where the epithelial cells are higher in the non-infected group. Total cells rise and remain high throughout the 96 hours. Neutrophils and macrophages both rise in the first 24 hours, but the neutrophils always remain the predominant cell type.

5.7 Chronic Lung Disease Vs No Chronic Lung Disease

5.7.1 Introduction

The data can be divided retrospectively for those infants that developed chronic lung disease and those that did not. Only infants who survived past 36 weeks were included in this analysis as those infants who died before this time could not have developed chronic lung disease by our definition. The pilot study (Chapter 4) found that infants that later went on to develop chronic lung disease had a significantly higher level of IL-8 at 24 hours post-natal age.

5.7.2 Results

5.7.3 Clinical Details

Table 5.4 gives the clinical details for infants divided into the group that developed chronic lung disease and the group that did not.

Table 5-4 Data are medians (range).

	Chronic Lung Disease	No CLD	p values
n	23	39	
Birthweight (g)	1032 (500 - 1680)	1175 (800 - 2300)	0.24
Gestation (wks)	29 (24 - 30)	29 (24 -30)	0.42
Length of Stay (days)	99 (63 - 185)	35 (2 -106)	**0.0000**
Time on Ventilator (days)	24 (3 - 86)	7 (1 - 45)	**0.0005**
Males	14	23	1.00
Ureaplasma	2	6	0.44
Ante-natal steroids	23	9	0.20
Chorioamnionitis	2	12	**0.033**
Surfactant	2	21	**0.004**
$AaDO_2$	25.4 (9.9 - 35.2)	21.9 (2.8 - 34.3)	**0.037**

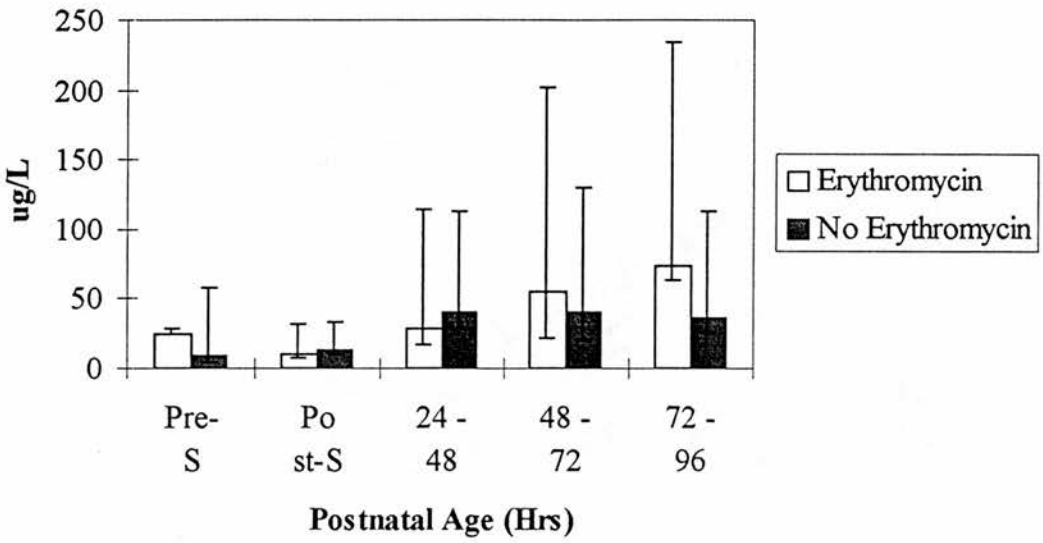
The significant differences between the two groups were length of stay and time ventilated. The group that developed chronic lung disease were more likely to have

received surfactant ($p < 0.005$). There was a significant difference ($p < 0.05$) for the mean arterial oxygen differences indicating that those infants that developed chronic lung disease had more severe lung damage than those not being ventilated.

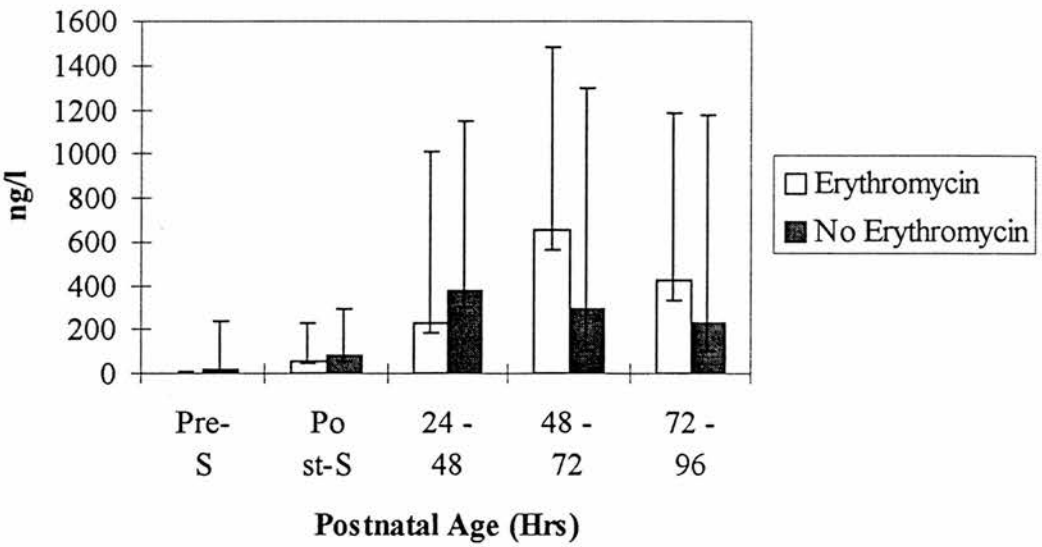
The difference in chorioamnionitis is significant, with those infants that later developed chronic lung disease being less likely to have had chorioamnionitis. The difference between the sexes is not significant.

Graphs 5.19 - 5.20: Cytokines in bronchoalveolar lavage comparing those that developed chronic lung disease with the group that did not. Data are medians and error bars are the interquartile range.

Graph 5.19: IL-8

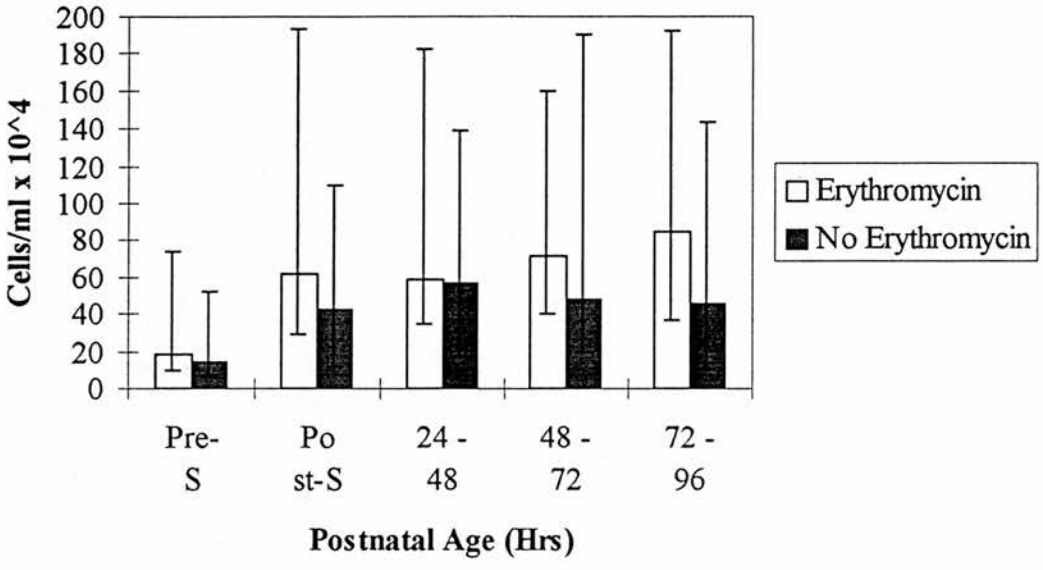


Graph 5.20: IL-1

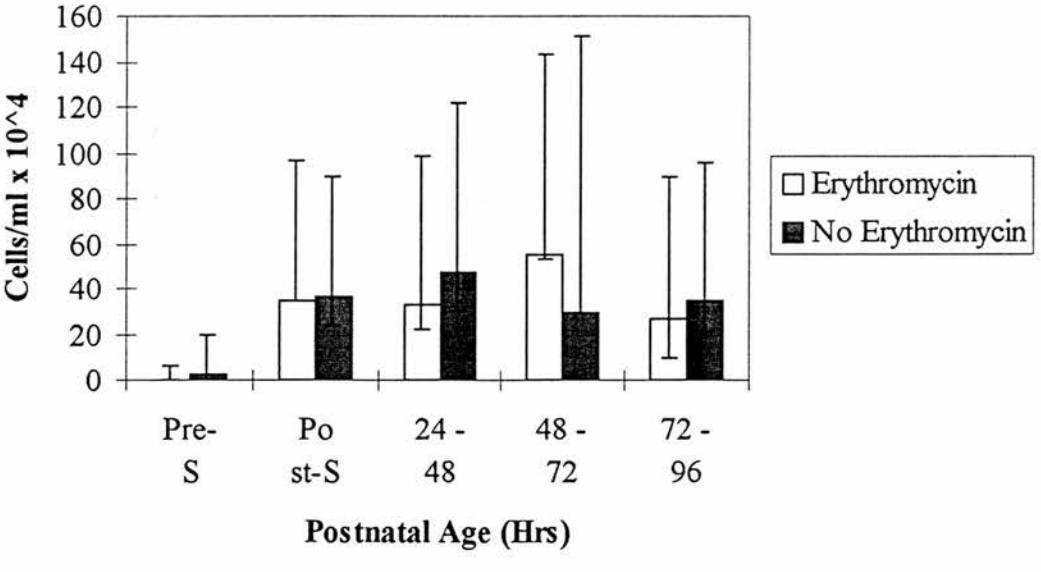


Graphs 5.21 and 5.22: Total cells and neutrophils in bronchoalveolar lavage comparing those that developed chronic lung disease with the group that did not. Data are medians and error bars are the interquartile range.

Graph 5.21: Total Cells

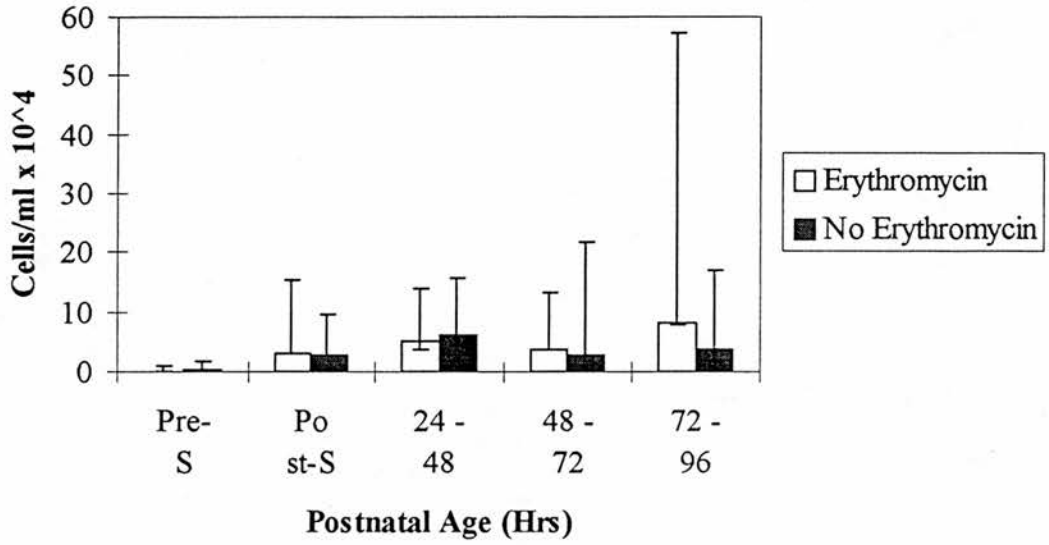


Graph 5.22: Neutrophils

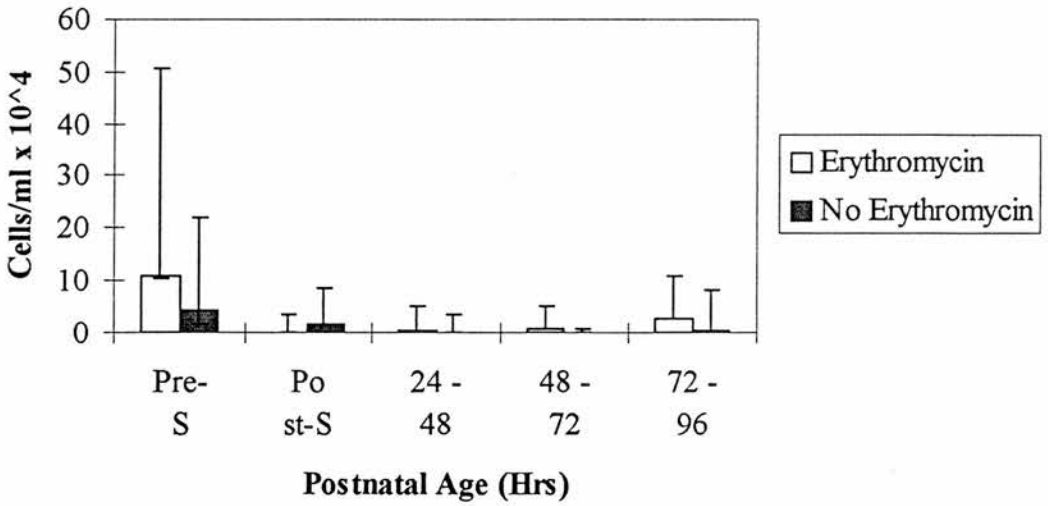


Graphs 5.23 and 5.24: Macrophages and epithelial cells in bronchoalveolar lavage comparing those that developed chronic lung disease with the group that did not. Data are medians and error bars are the interquartile range.

Graph 5.23: Macrophages



Graph 5.24: Epithelial Cells



IL-8 and IL-1β are significantly higher ($p < 0.003$ and $p < 0.05$ respectively) on admission in the group that did not develop chronic lung disease. When the data is analysed using a multiple linear regression analysis which takes into account other factors such as caesarean section, chorioamnionitis, birthweight and gestation the differences are no longer significant and the only factor which has a positive correlation with the initially high IL-8 and IL-1β is chorioamnionitis ($p < 0.001$).
The TNF-α does not show any significant differences between the two groups and the median values for all time periods was 0. For this reason the data is not graphed.

There were no significant differences between the cells, total and each cell type at any time point. The patterns are similar for both groups and they are the same as the patterns for all infants.

5.8 Surfactant Vs No Surfactant

5.8.1 Introduction

At the time of the study infants that received surfactant were given Curosurf or Exosurf. A randomised trial of Curosurf vs Exosurf was also taking place within our unit and many of the infants that were enrolled on the erythromycin trial were also enrolled on that study. The data presented here for the erythromycin trial does not distinguish between the two surfactants.

Cytokine and cell data were collected for many of the infants enrolled on the surfactant trial and it is presented here as a comparison of samples immediately pre and post surfactant. We wished to investigate whether Curosurf - which is natural and has proteins - or Exosurf - which is wholly synthetic- provoked a different initial inflammatory response.

5.8.2 Results for the Erythromycin Trial

5.8.3 Clinical Details

Table 5.5 gives the clinical details for infants that received surfactant and those that did not.

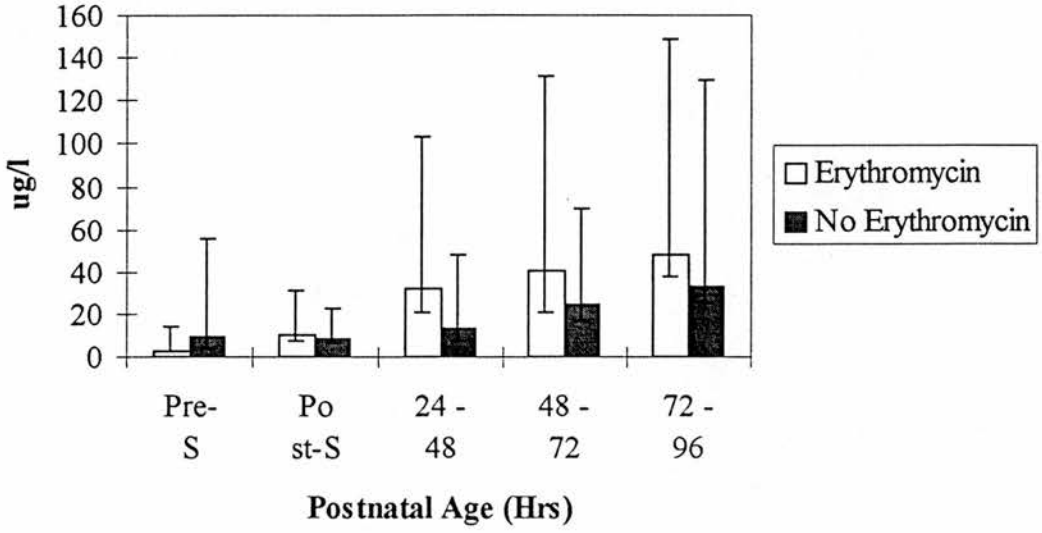
Table 5-5: Data are medians (range).

	Surfactant	No Surfactant	p values
n	51	24	
Birthweight (g)	1045 (500 - 1820)	1020 (640 - 2300)	0.37
Gestation (wks)	29 (23 - 30)	28 (24 - 30)	0.43
Length of Stay (days)	51 (2 - 214)	34 (1 - 122)	0.061
Time on Ventilator (days)	6 (2 - 63)	2.5 (1 - 80)	**0.0009**
CLD (O_2 at 36wks)	22	2	**0.002**
Males	26	12	1.00
Ante-natal steroids	23	9	0.79
Chorioamnionitis	7	8	0.06
Ureaplasma	4	4	0.08
Died	8	5	1.00
$AaDO_2$	24.2 (2.8 - 35.2)	22.2 (11.2 - 31.9)	0.26

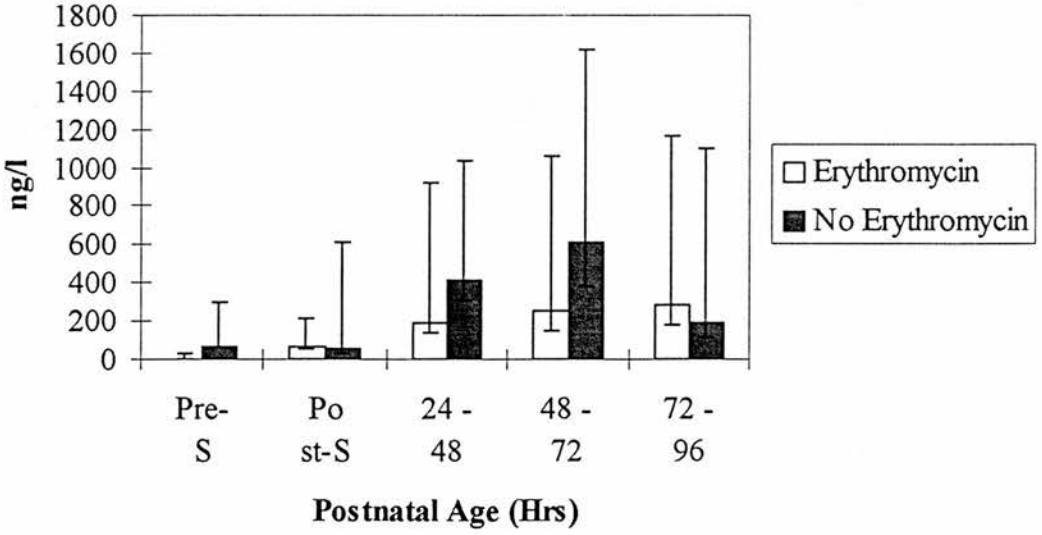
Infants that received surfactant had a longer time on the ventilator and were more likely to develop chronic lung disease.

Graphs 5.25 - 5.26: Cytokines in bronchoalveolar lavage comparing the group that received surfactant with the group that did not. Data are medians and error bars are the interquartile range.

Graph 5.25: IL-8

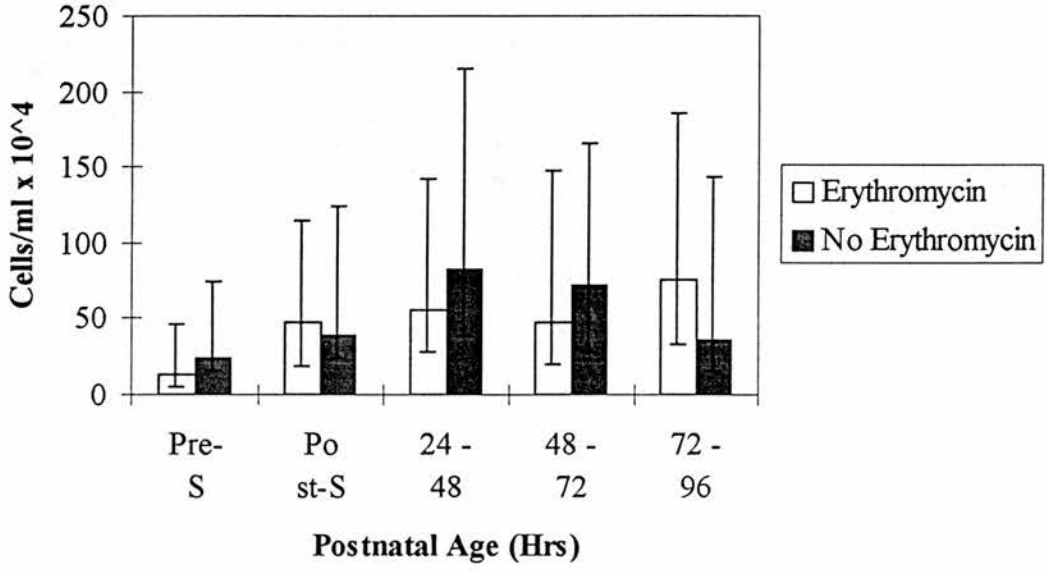


Graph 5.26: IL-1

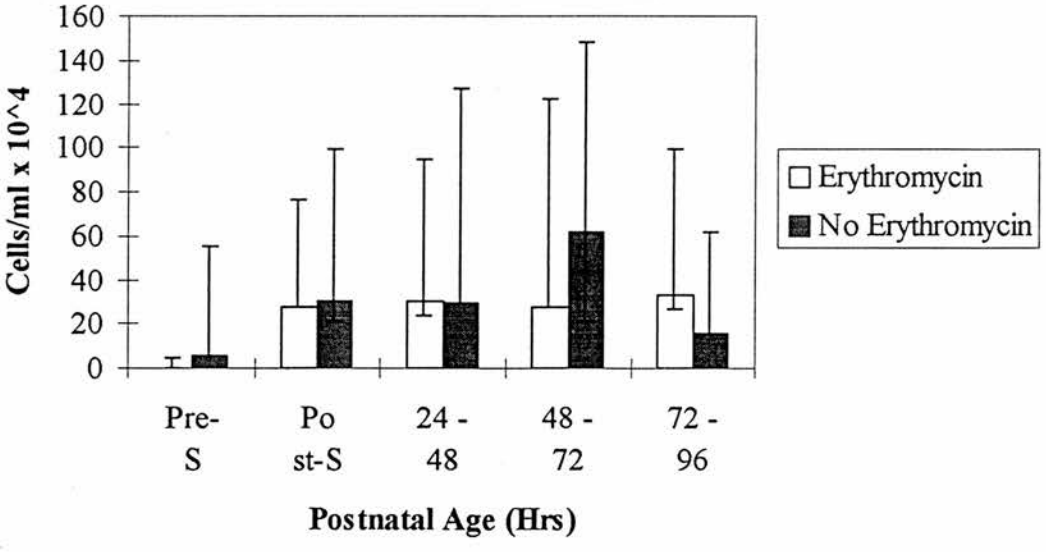


Graphs 5.27 and 5.28: Total cells and neutrophils in bronchoalveolar lavage comparing the group that received surfactant with the group that did not. Data are medians and error bars are the interquartile range.

Graph 5.27: Total Cells

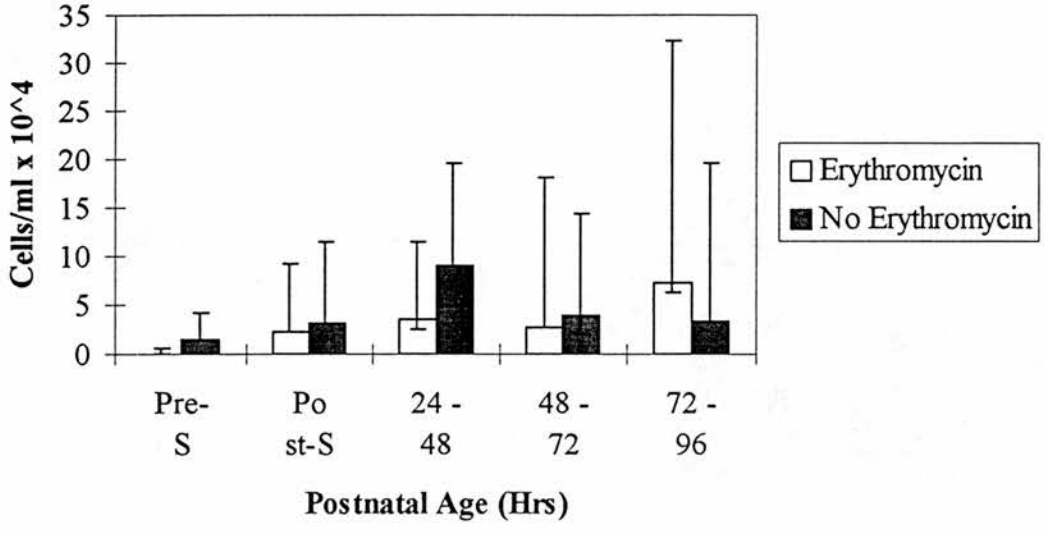


Graph 5.28: Neutrophils

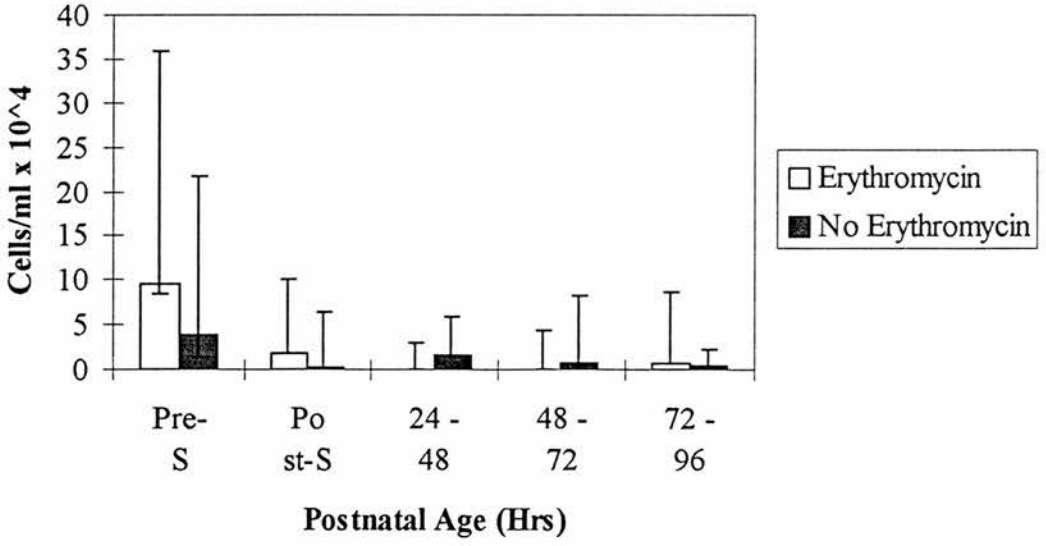


Graphs 5.29 and 5.30: Macrophages and epithelial cells in bronchoalveolar lavage comparing the group that received surfactant with the group that did not. Data are medians and error bars are the interquartile range.

Graph 5.29: Macrophages



Graph 5.30: Epithelial Cells



IL-8, IL-1β, neutrophils and macrophages are significantly different on admission and are lower for those infants receiving surfactant, but these values are lower before surfactant administration, and are therefore unrelated to it. The TNF-α is lower after surfactant administration but only at $p < 0.05$. So the infants that receive surfactant had lower early cytokine and inflammatory cells on admission, but significantly more of them developed chronic lung disease. The group of infants that had higher early cytokines had a positive correlation with histological chorioamnionitis ($p < 0.01$), and in a multiple linear regression taking account of this there was no differences between the two groups pre-surfactant cytokine levels were zero.

5.8.4 Results of the Different Surfactants

5.8.5 Study Group

23 ventilated newborn infants on the first day of life with respiratory distress syndrome and an A/a ratio < 0.22 were randomised to receive Curosurf or Exosurf. Samples were bronchoalveolar lavage fluid taken immediately prior to and 24 hours post-surfactant administration.

Table 5-6: Clinical Details. Data are medians (range) or number.

	All Infants	**Curosurf**	**Exosurf**
n	23	12	11
Gestation (wks)	29 (26 - 32)	29 (26 - 32)	29 (27 - 32)
Birthweight (g)	1450 (755 - 2345)	1395 (755 - 2320)	1543 (880 - 2345)
Prenatal steroids	12	6	6
Male	14	9	5

There were no significant differences between the groups that received Curosurf or Exosurf by Mann-Whitney U Test.

5.8.6 Cytokine and Cell Data

Table 5-7: Cytokine Levels and Cell Differential Counts Pre vs Post Surfactant Treatment. Data are medians.

	All Infants	**Exosurf**	**Curosurf**
IL-8 ng/ml	4.4 vs 20.7 (p = 0.078)	4.2 vs 23.7 **(p = 0.04)**	6.2 vs 15.6 (p = 0.58)
TNF pg/ml	0 vs 0 (p = 0.29)	0 vs 0 (p = 1.0)	0 vs 0 (p = 0.28)
% neutrophils	2 vs 77 **(p = 0.001)**	22 vs 75 (p = 0.06)	1 vs 77 **(p = 0.01)**
% macrophages	0 vs 5 (p = 0.13)	6 vs 5 (p = 0.76)	0 vs 8 (p = 0.10)

Statistics were performed with a Wilcoxon Signed Ranks test.

TNF-α was not detectable in either group, but IL-8 rose after surfactant in both groups. The infants that received Exosurf showed a greater rise that was statistically significant. The percentage of the total cells that were neutrophils also rose in both groups and at 24 hours the levels were similar. However the rise in those infants that received Curosurf was significant at p = 0.01, whereas the rise for Exosurf did not reach statistical significance. Both groups had low macrophage numbers over the 24 hour period.

5.9 Discussion

Infants ≤ 30 weeks gestation on ventilatory support in our unit showed an inflammatory response, measured by IL-1β and IL-8, which rose and remained high over the first 96 hours of life. In contrast, TNF-α was undetectable in most samples. The cellular response in the lungs showed a neutrophil influx at 24 hours with the neutrophils remaining the predominant cell type. Macrophage numbers also rose but never constituted more than 15% of the total cell population.

Ante-natal steroids in recent years have become a very important treatment to promote maturation of the lungs *in utero*. They also have known anti-inflammatory properties, but had no effect on the inflammatory response in this study nor did they correlate with an improved outcome. Infants that received steroids were equally likely to have received surfactant and be colonised by the genital mycoplasmas, but less likely to have had chorioamnionitis. Chorioamnionitis did not correlate with colonisation with the genital mycoplasmas.

High IL-1β and IL-8 in the admission sample correlated significantly with histological chorioamnionitis ($p < 0.0001$; Mann-Whitney U test), which has also been reported elsewhere (Watterberg *et al.*,1996).

Infants at ≤ 30 weeks gestation with a diagnosis of severe respiratory distress syndrome (RDS) due to immaturity of the lungs and surfactant deficiency were given exogenous surfactant. Around 20% of infants with RDS can be expected to develop chronic lung disease (Verma.,1995). Infants without RDS had a significantly higher IL-1β, IL-8, neutrophil and macrophage cell counts on admission which correlated with chorioamnionitis. Normal lung development is under the control of glucocorticoids which act directly at the protein synthesis level to induce surfactant production (Gross.,1990). IL-1β can directly stimulate the production of corticotrophin-releasing hormone (Sapolsky *et al.*,1987) which in turn stimulates the production of several hormones including glucocorticoids (Bernton *et al.*,1987). Thus infants *in utero* with chorioamnionitis may have surfactant production stimulated earlier than those without chorioamnionitis and be less likely to have RDS. This was also shown in a recent study by Watterberg *et al* 1996, though their infants with

chorioamnionitis were more likely to developed chronic lung disease, which is not shown in our study. Watterbergs study had small numbers, and were defining chronic lung disease as oxygen dependence at 30 days - which others does not correlate well with outcome (Shennan *et al.*,1988). In our study, of infants with chorioamnionitis, 12/15 were in oxygen at 28 days but this was not significantly different from the non-chorioamnionitis group ($p = 0.06$). Only 2/14 infants went on to develop chronic lung disease defined by oxygen dependence at 36 weeks and this was significant ($p = 0.003$). In our study infants that developed chronic lung disease were more likely to have had RDS. Giving surfactant had no effect on the cytokine response at this early stage, although there was a trend in a smaller sub-group that received Exosurf to have a larger rise in IL-8 over the first 24 hours compared to Curosurf. The group that received surfactant did go on to develop chronic lung disease more often than those infants not having chronic lung disease, but this did not correlate with an inflammatory response.

Infection with the genital mycoplasmas did not increase the early inflammatory response in the lung nor did it correlate with incidence or severity of chronic lung disease. The infection rate (13%) was lower compared to other clinical studies where rates have been 17 - 30% for *U. urealyticum* isolated from bronchoalveolar lavage samples (Payne *et al.*,1991; Wang *et al.*,1988; Cassell *et al.*,1988). All of these studies showed a correlation between *U. urealyticum* colonisation and the development of chronic lung disease. Ours was a relatively large study and in addition to culture used PCR to identify *U. urealyticum* so this is likely to be a true reflection of the infection rate in the unit.

As the colonisation with the genital mycoplasmas did not significantly alter the clinical course of the infants, treatment with erythromycin could not have had any anti-bacterial effect.

Erythromycin is readily internalised by phagocytes in an energy (Viggiano *et al.*,1994) and calcium ion dependent (Rowen *et al.*,1995) manner, and phagocytosis of particles increases its intracellular concentration (Santoro *et al.*,1995). It has been suggested that its immune suppression is related to its internalisation which affects neutrophil

chemotaxis (Nelson *et al.*,1987; Esterley *et al.*,1990), elastolytic properties, (Ichikawa *et al.*,1992) and apoptosis (Aoshiba *et al.*,1997). Preterm neonatal phagocytes have less Fc receptors on their cell surface compared to adults (Carr *et al.*,1992a; Falconer *et al.*,1995a) and these receptors are very important in chemotaxis and phagocytosis. Studies have suggested that in neonates both these functions of neutrophils are deficient (Eisenfeld *et al.*,1994; Falconer *et al.*,1995; Hill.,1987). If neonatal neutrophils were unable to internalise erythromycin then this might explain why the reported anti-inflammatory effects in adult airway diseases (Esterley *et al.*,1990; Ichikawa *et al.*,1992; Oishi *et al.*,1994) were not duplicated here.

Our initial pilot study investigating IL-8 in the development of chronic lung disease showed that at 24 hours postnatal age infants that later developed chronic lung disease (defined as an oxygen requirement at 28 days) had a higher IL-8 level than those that did not develop chronic lung disease. In this larger study group the pilot study findings were not confirmed. Infants who did not later develop chronic lung disease were admitted to the unit with a higher level of IL-8 and IL-1β which correlated very strongly with histological chorioamnionitis. Although previous studies have shown that isolation of *U. urealyticum* correlates with chorioamnionitis (Wang *et al.*,1993), it did not in our study. We did not culture the placenta for genital mycoplasmas.

5.10 Conclusions

The early inflammatory response is not altered by infection with the genital mycoplasmas, treatment with erythromycin or surfactant. The incidence of chronic lung disease and its severity, as measured by alveolar arterial oxygen difference, were also unrelated to the early pro-inflammatory cytokines, or cellular response.

6. Cell Staining

6.1 Introduction

In chronic lung disease it has been speculated that immune cells that have migrated from the peripheral circulation into the lung may be important in disease progression (Ogden *et al.*,1984; Merrit *et al.*,1981). There is disagreement between studies with some suggesting only the presence of neutrophils contributes to the outcome (Arnon *et al.*,1993). The roles of neutrophils and macrophages are vastly different in the progression of an inflammatory response with only macrophages having a role in the development of pulmonary fibrosis.

In our samples of bronchoalveolar lavage we would expect to find epithelial and immune type cells and possibly red blood cells depending on the degree of lung damage. Most reported studies have used a standard differential stain to distinguish cells based on their morphology, where mature neutrophils are easily recognised but monocytes/macrophages are not. A recent paper (Murch *et al.*,1996b) has suggested that because there is great phenotypic differences between cells of the monocyte/macrophage lineage identification by routine staining is difficult. The use of more specific immunocytochemical staining methods is required to establish the cell types.

6.1.1 Neutrophil Maturation

Neutrophils differentiate in the blood marrow from precursors common to all haematopoietic cells. They undergo six distinct developmental stages the penultimate of which are band cells which have an unsegmented horse-shoe shaped nucleus and in this respect they are not unlike monocytes in appearance. The last two stages are held in a large reserve pool within the bone marrow and are normally released upon stimulation. The band cells do not normally constitute more than 10% of the circulating neutrophil population (Balado.,1996), but under stress and a high demand for neutrophils a greater proportion of the cells will be less mature. Premature newborns are particularly vulnerable to infections due to a diminished storage pool

and this is exacerbated by their neutrophils having an impaired ability to migrate to the site of infection (Eizioni.,1994).

6.1.2 Differential Stain

Differential staining relies on the properties of cells organelles and proteins to acquire colour in the presence of reagents, but also requires the observer to distinguish morphological features distinctive to cell types. Cells of the immune system can be separated using eosin and a thiazine dye, which results in the nuclei of neutrophils staining purple and cytoplasm staining pink. Further morphological features of neutrophils are granules in the cytoplasm and the multi-lobed nucleus. Cells of the monocyte/macrophage lineage stain blue in the nuclei and pale blue in the cytoplasm. The nuclei is characteristically kidney shaped and if the cells are active, the cytoplasm can be well spread and vacuoulated. With this stain red blood cells appear pale pink and are characteristically non-nucleated and indented, whereas epithelial cells have a dense blue nuclei and pale blue, well spread, smooth cytoplasm (See Photos 6.1 and 6.2).

6.1.3 Immunocytochemical Stain

Immunocytochemistry exploits the different cell surface proteins which appear uniquely on different cell types. Each of these cell specific proteins can be targeted by monoclonal antibodies and by coupling the monoclonal to a specific label the targeted cell can be visualised.

There are several immunocytochemical techniques commonly used, but due to an abundance of endogenous peroxidase in haematological preparations the alkaline-phosphatase anti-alkaline-phosphatase or APAAP technique is the method of choice. It can be applied in many successive layers and as such is very sensitive. It involves the application of a primary monoclonal antibody which binds specifically to a protein found on the surface of the target cell. A second antibody which has been raised against the species of the primary antibody is then added and binds to the primary antibody. The next stage adds the APAAP antibody which is the same species as the primary antibody and to which alkaline phosphatase enzyme has been

Photo 6-1: Differential Stain (Diff-Quik) x 200.

Typical bronchoalveolar lavage sample preparation with distinct cell types seen and background mucous (Mc). Red blood cells appear small, with no nucleus or internal structures (r). Individual cell types are difficult to distinguish at this magnification, but some macrophages (M) and neutrophils (N) are visible.

Photo 6-2: Differential Stain (Diff-Quik) x 400 oil immersion.

Above sample at a higher magnification. The cell morphology is more easily made out. Macrophages/monocytes (M) clearly have darker cytoplasm and a single kidney shaped nucleus. Neutrophils (N) have a lighter cytoplasm and multi-lobed nucleus. The neutrophil cytoplasm looks pinker with the naked eye, the blue colour is due to a blue filter required to take sharp pictures at this magnification.

Photo 6.1: Differential Stain (Diff-Quik) x 200.

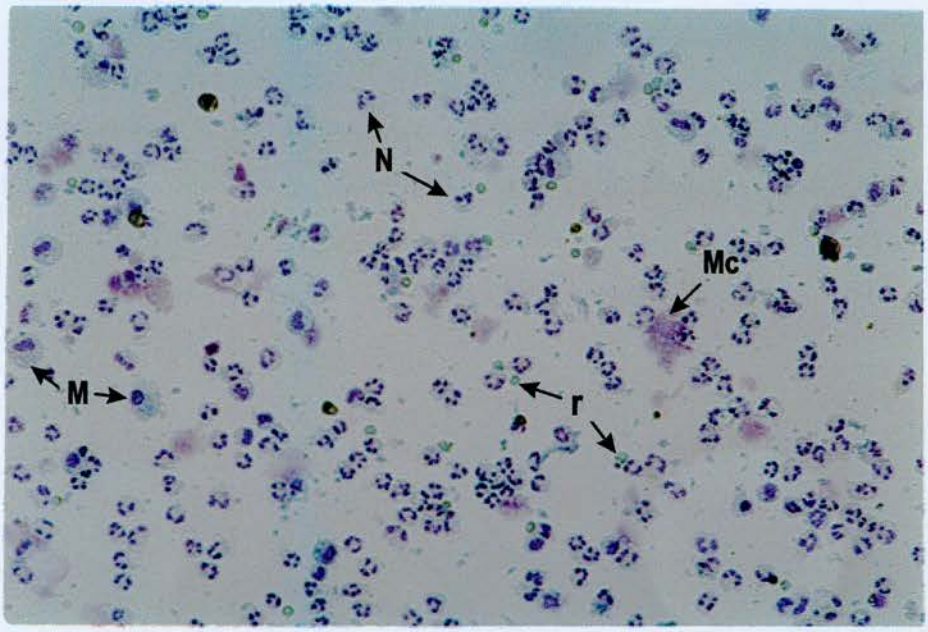
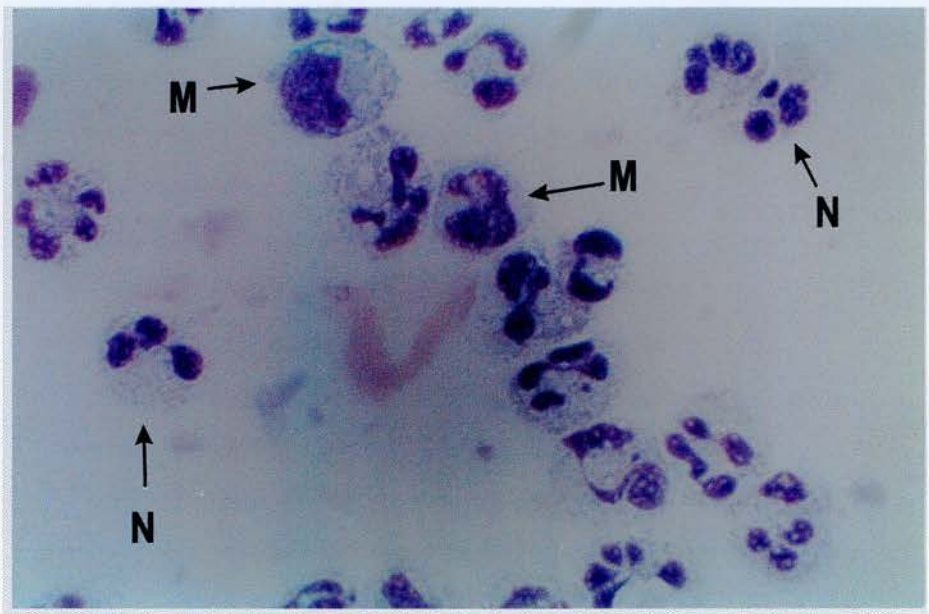


Photo 6.2: Differential Stain (Diff-Quik) x 400 oil immersion.



conjugated. The APAAP antibody binds to the second antibody to create a sandwich (See Figure 6.1). With the addition of fast red substrate red colour is produced at the site of the APAAP sandwich and positive cells are easily recognised using light microscopy.

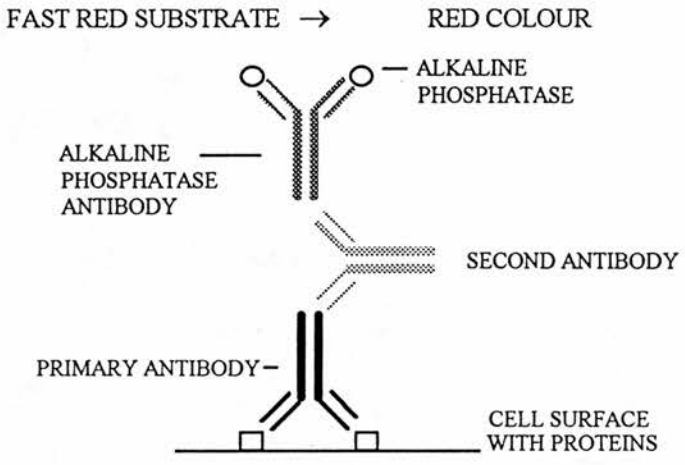


Figure 6-1: Diagram of the APAAP sandwich

This staining method gives a more discriminatory method of separating cell types which are difficult to distinguish via staining and morphology alone. It should be more accurate and less observer dependent.

6.1.4 Cells Surface Proteins

Cells of the monocyte/macrophage can be identified by the CD68 antigen cluster which has a molecular weight of 110kD and is probably associated with lysosomal granules. It is distributed primarily in the monocyte/myeloid cells though it will stain neutrophils weakly (Pulford *et al.*,1990).

Neutrophils display several different characteristics which allow them to be identified. They contain a neutral protease found primarily in the azurophilic granules but which is also present in small amounts in some human monocytes (manufacturers specification sheet). This means there is some cross-reactivity with cells of the

monocyte/macrophage lineage and so the anti-human neutrophil elastase antibody is not ideal.

Sigma have recently produced a PMN-specific antibody with no known cross-reaction to monocyte/macrophages. It detects polymorphonuclear (PMN)-specific membrane antigens and was used when it became available.

The epithelial cells can be stained for cytokeratin, the intermediate filaments which are associated with the cytoskeleton. However, as slide numbers were limited it was decided to concentrate on the cells of the immune system.

The two techniques were compared to establish whether differential staining is adequate to distinguish between cell types.

6.2 Preparation of Test Slides

A buffy coat (a white blood cell enriched pack from South East Blood Transfusion Service, Edinburgh) was diluted 1:1 with RPMI-1640 and 25 mls carefully layered onto 25mls of Histopaque 1077 (Sigma, Cat. No. 1077-1) in a 50 ml centrifuge tube. This was then centrifuged at 400g for 30 mins (Jouan) ensuring the brake was switched off. The layer of white blood cells at the interface of the Histopaque and the plasma was carefully removed with a pasteur pipette and washed three times with RPMI-1640. Cells were then counted and viability assessed using trypan blue exclusion (viability > 98%).

The cell concentration was adjusted to 4 x 10^5 cells/ml in RPMI-1640 and 100µl of this was added to slides previously wiped with alcohol in the cytospin (Shandon Cytospin 3). They were spun at 1000 rpm for 3 mins and treated as below depending on the staining method.

6.3 Differential Stain on Test Slide

6.3.1 Materials

Diff-Quick Staining Kit, Dade containing methanol as a fixative, eosin and a thiazine dye.

Distilled water.

6.3.2 Method

The slide was air dried and stained differentially by sequentially dipping the slide 5 times for 1 second each into first methanol, then the eosin and finally the thiazine dye. The slide was washed in distilled water and left to air dry before counting 300 cells.

6.4 Immunocytochemical Staining on Test Slide

Slides were air dried overnight and then stored at -20°C with silicon gel packets to act as a desiccant until stained.

A standard APAAP technique was used to determine the optimum primary antibody concentration for each antibody.

6.4.1 Materials

Acid hemallum stain (see Appendix A: Solutions)

Acetone

Tris buffered saline (TBS) (see Appendix A: Solutions)

Normal human serum

Substrate (see Appendix A: Solutions)

Aquamount (BDH, Cat. No. 36626)

RPMI-1640 without phenol red (SE-BTS, Edinburgh)

Trypan blue (Sigma, Cat. No. T8154)

Second antibody: 50µl rabbit anti-mouse immunoglobulins (Dako, Cat. No. Z259) + 100µl normal human serum + 2.5 mls TBS

Alkaline phosphatase anti-alkaline-phosphatase (APAAP): 100µl APAAP (Dako, Cat. No. D651) + 2.5 mls TBS

6.4.2 Primary Antibodies

Anti-CD68 (Dako, Cat. No. M718). Diluted in TBS to 1:100, 1:150 and 1:200.

Anti-human neutrophil elastase (Dako, Cat. No. NP57). Diluted in TBS to 1:200, 1:250 and 1:300

Anti-human neutrophils (Sigma, Cat. No. N1765). Diluted in TBS to 1:50, 1:100, 1:200, 1:300 and 1:400.

Control primary antibody: IgG class 1 mouse anti-human thyroid stimulating hormone (SAPU). Diluted in to TBS 1:50, 1:100, 1:200, 1:300 and 1:400.

6.4.3 Method

Slides were selected for staining and scored round the smear using a diamond pen. They were allowed to come to room temperature for at least 10 minutes before being fixed in acetone for 15 minutes. The slides were not allowed to dry out from this point on and all incubations were carried out at room temperature in a humidified box.

Slides were washed in TBS and dried off round the smear. This drying step was carried out after every wash with TBS because if the slide is too wet the antibody will diffuse over the slide and the antibody concentration in contact with the smear will be too low. 100µl of primary antibody was gently dropped onto the smear and incubated for 1 hour. It was washed in TBS before addition of 100µl second antibody and incubated for 30 minutes. After washing in TBS 100µL of APAAP was added and incubated for 30 minutes. Again it was washed in TBS and another 100µl of second antibody was added and incubated for 20 minutes. The slide was washed with TBS and 100µl APAAP was added for 20 minutes. After washing in TBS the substrate was filtered (Whatman Filter No.1) onto the smear and the colour development watched. After sufficient colour development (~15 minutes) the slide was placed in running tap

water for 5 minutes, acid hemallum for 5 minutes and then back to running water for 5 minutes before being mounted in aquamount. After 24 hours to harden it was viewed under oil immersion and counts were made on 300 cells.

6.4.4 Results

Table 6.1 details the results for the differential stain which are approximately normal values for a healthy adult.

The CD68 antibody results showed little difference between the three antibody concentrations except that the 1:100 dilution was clearly the first to develop colour and stained the strongest. The cell counts in Table 6.2 are therefore given at that concentration. Background was low for all slides.

The neutrophil elastase antibody had high background staining at 1:100 but very little other differences between antibody concentrations. The 1:300 dilution showed the strongest and most discrete staining and so Table 6.2 shows results for that dilution.

The anti-PMN antibody showed cell specific staining for all slides and all slides except the 1:50 had low background staining. The 1:100, and 1:200 dilution were too intense and 1:400 too pale. 1:300 results are given in Table 6.2

For the control antibody (anti-TSH) no cell specific staining was seen at any of the dilutions and this antibody will therefore be a suitable control. There is also no endogenous alkaline phosphatase activity.

Table 6-1: Results of counting 300 cells stained differentially

Cell Type	**Percentage**
Neutrophils	60
Monocytes	25
Lymphocytes	15

Table 6-2: Results of counting 300 cells stained immunocytochemically.

	CD-68 (monocyte/ macrophage)	Neutrophil elastase	Anti-PMN
Red	32	60	54
No Stain	78	40	46

6.4.5 Discussion

Both the neutrophil antibodies are very similar to each other and indeed to the differential stain. This is not unexpected as these are white blood cells from healthy donors and as such make an easy population of cells to discriminate from other cell types. There are no immature nor abnormal cells within this population.

The differential count discriminates between lymphocytes and monocytes based on size and these can be very difficult to distinguish. This may be why there are slightly higher macrophage numbers using the CD-68 stain.

The two techniques measure the same on these test slides of healthy cells.

6.5 Immunocytochemical Stain vs. Differential Stain on Bronchoalveolar Lavage Samples

Bronchoalveolar lavage samples were prepared onto cytospin slides as previously described (see "Chapter 2 p30"), air dried for 24 hours and frozen at -20°C with desiccant until use. Sample size for these infants are small as are cell numbers and in many cases it was not always possible to get enough slides to do a comparison. A minimum of four slides was required, one for differential stain, one for CD68 stain, one for neutrophil antigens and a control stain. As the identification of monocytes/macrophages was under dispute it was decided that if only three slides were available after the differential stain, the CD68 antigen would be stained for and neutrophil staining would be omitted.

6.5.1 Materials and Methods

Both methods are as described above. For differential stain see "Method p119" and for immunocytochemical stain see "Method p120".

6.5.2 Results

Some slides did not survive the freezing process over a period of time, mostly those that were frozen for > 2 years. This may have been due freezer breakdowns, thus causing repeated freeze/thaws, or may be that over that period of time the cells deteriorate naturally. Of the slides that did survive there were 60 available for CD-68 analysis and 32 available for neutrophil analysis.

Photos 6.3 - 6.7 give typical examples of each of the samples.

The results were plotted on scatter plots Graphs 6.1 and 6.2 and the correlation coefficient (r) is given.

Photo 6-3: Anti-TSH antibody x 200.
Control antibody stains no cells.

Photo 6-4: Anti-CD68 x 400 oil immersion.
The cytoplasm of the macrophage stains bright red (M) compared to the unstained neutrophils and epithelial cells (U). Mucous (Mc) contributes to the background colour.

Photo 6.3: Anti-TSH antibody x 200.

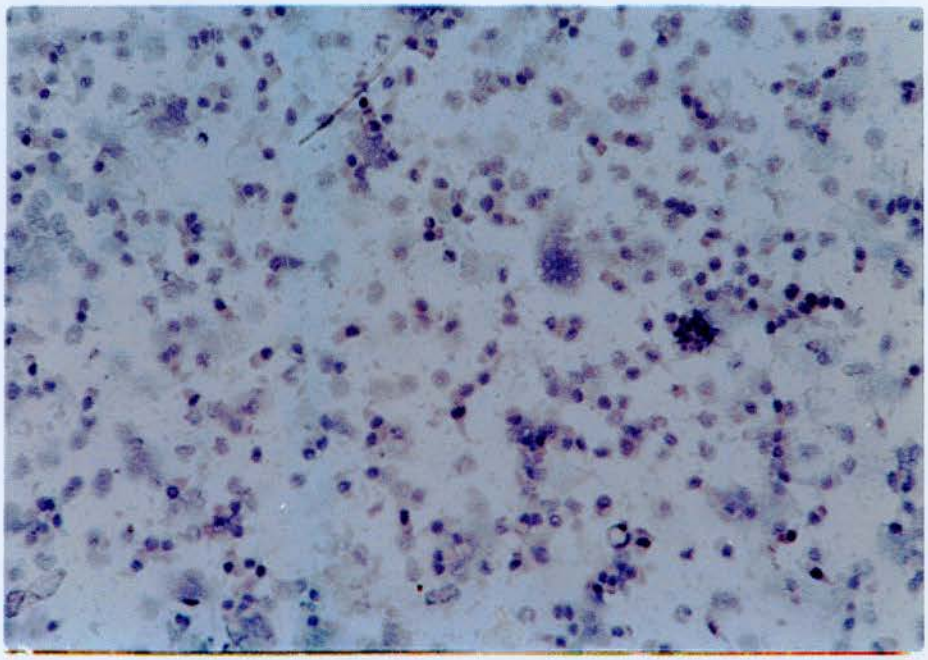


Photo 6.4: Anti-CD68 x 400 oil immersion.

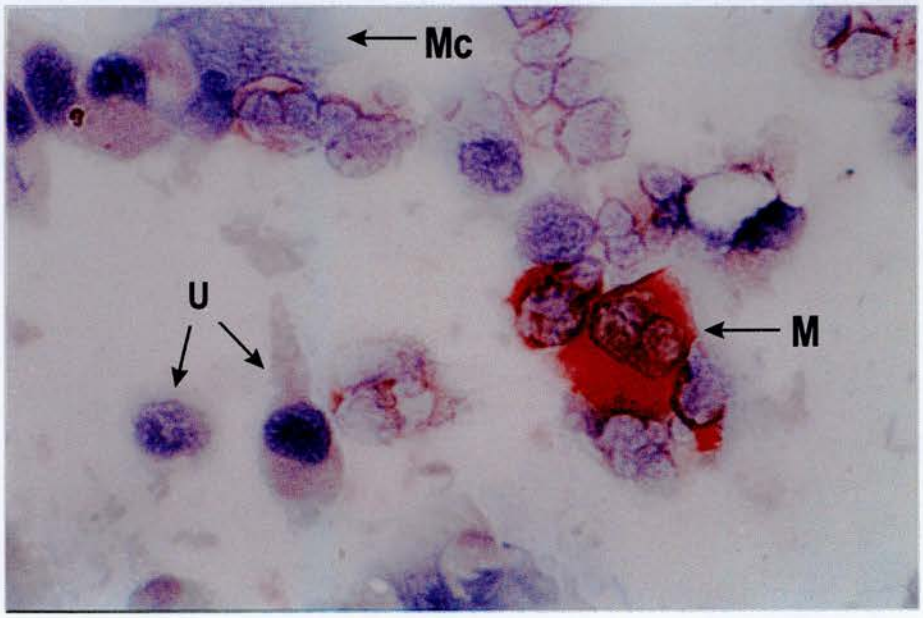


Photo 6-5: Anti-neutrophil elastase x 200.

This photo gives an overview of a typical sample, where the red-stained neutrophils are clearly distinguishable from the blue negative cells.

Photo 6-6: Anti-neutrophil elastase x 400 oil immersion.

This above photo at higher magnification shows the strongly staining red neutrophils (N) and the unstained macrophages and epithelial cells (U). The mucous (Mc) also stains slightly red.

Photo 6.5: Anti-neutrophil elastase x 200.

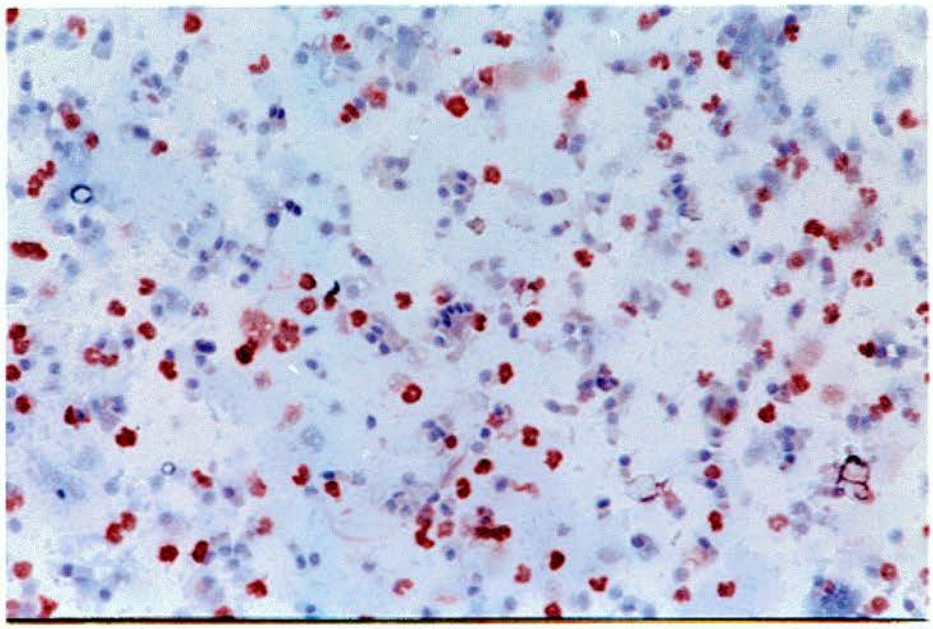


Photo 6.6: Anti-neutrophil elastase x 400 oil immersion.

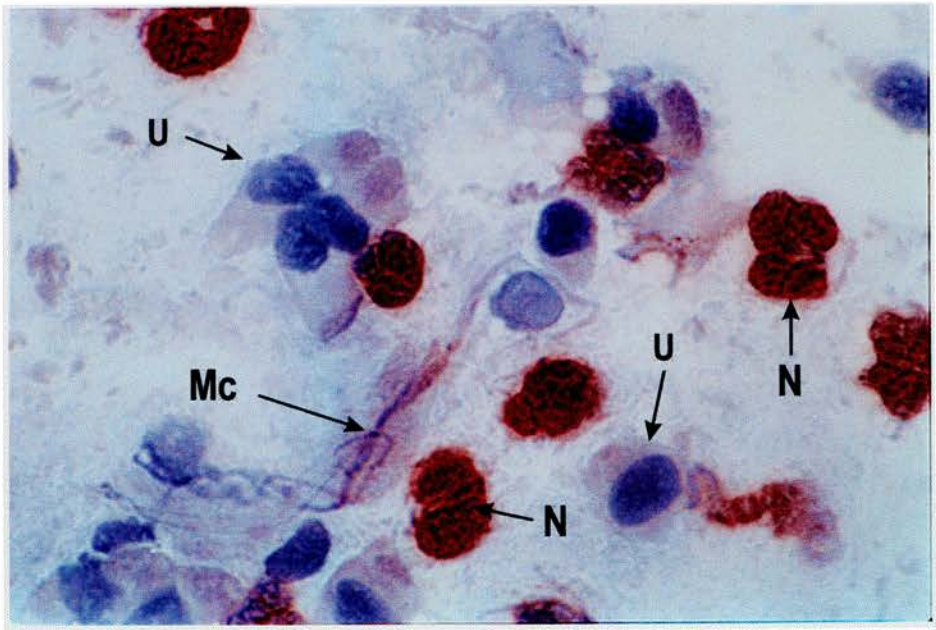
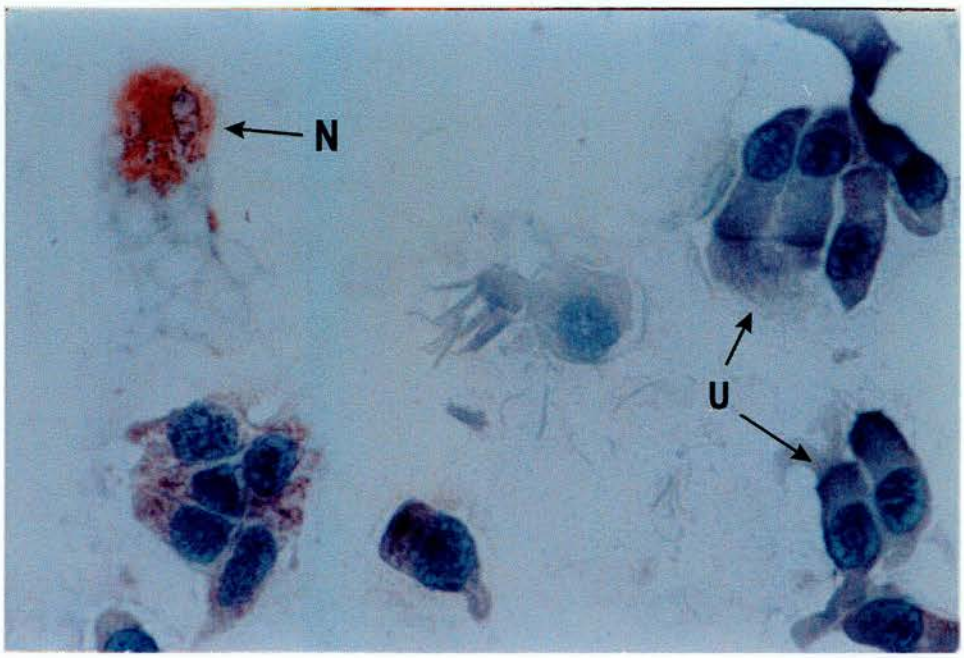


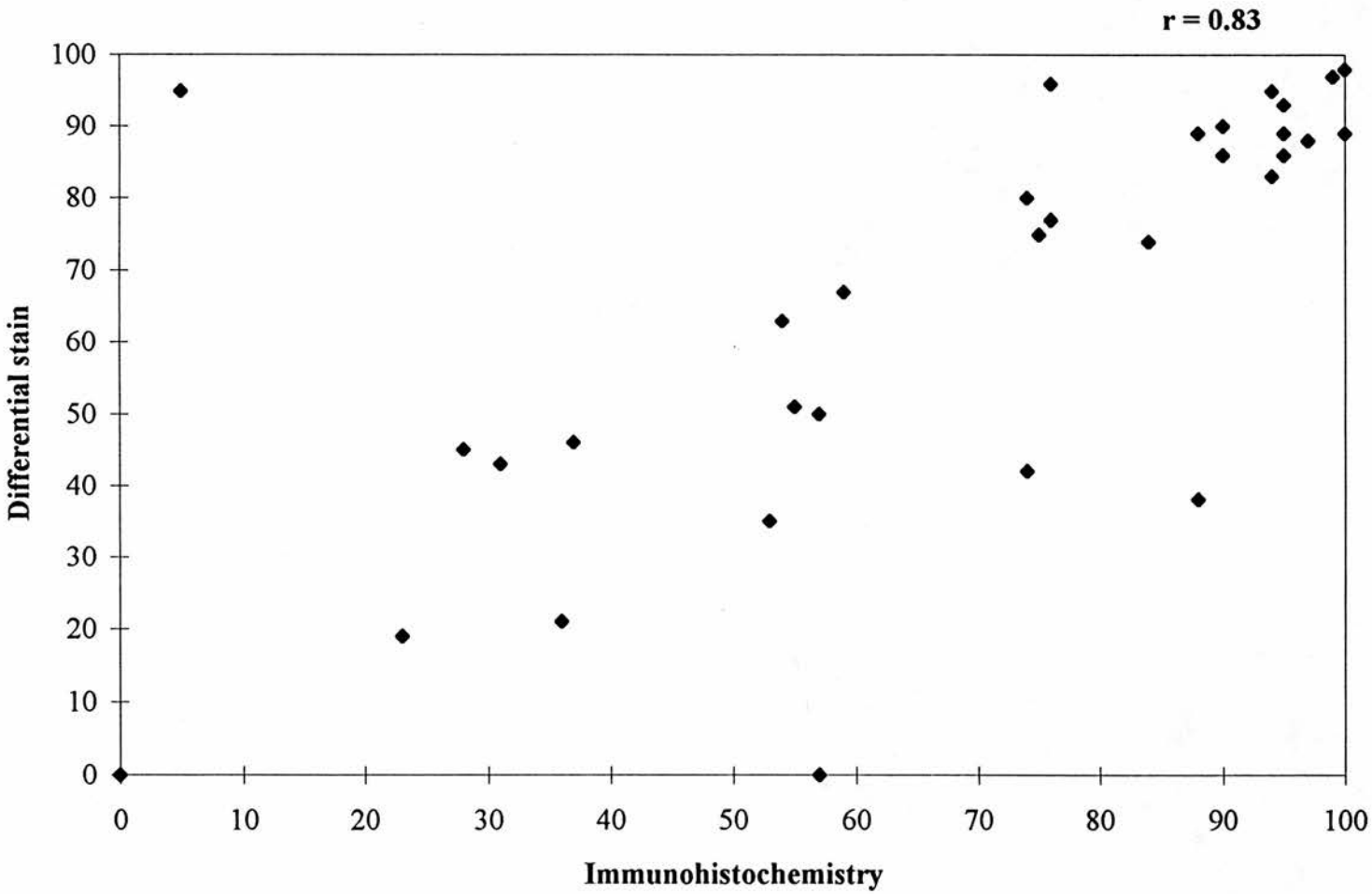
Photo 6-7: Anti-PMN x 400 oil immersion.

The neutrophil (N) stains red, but the cuboidal epithelial cells are unstained (U).

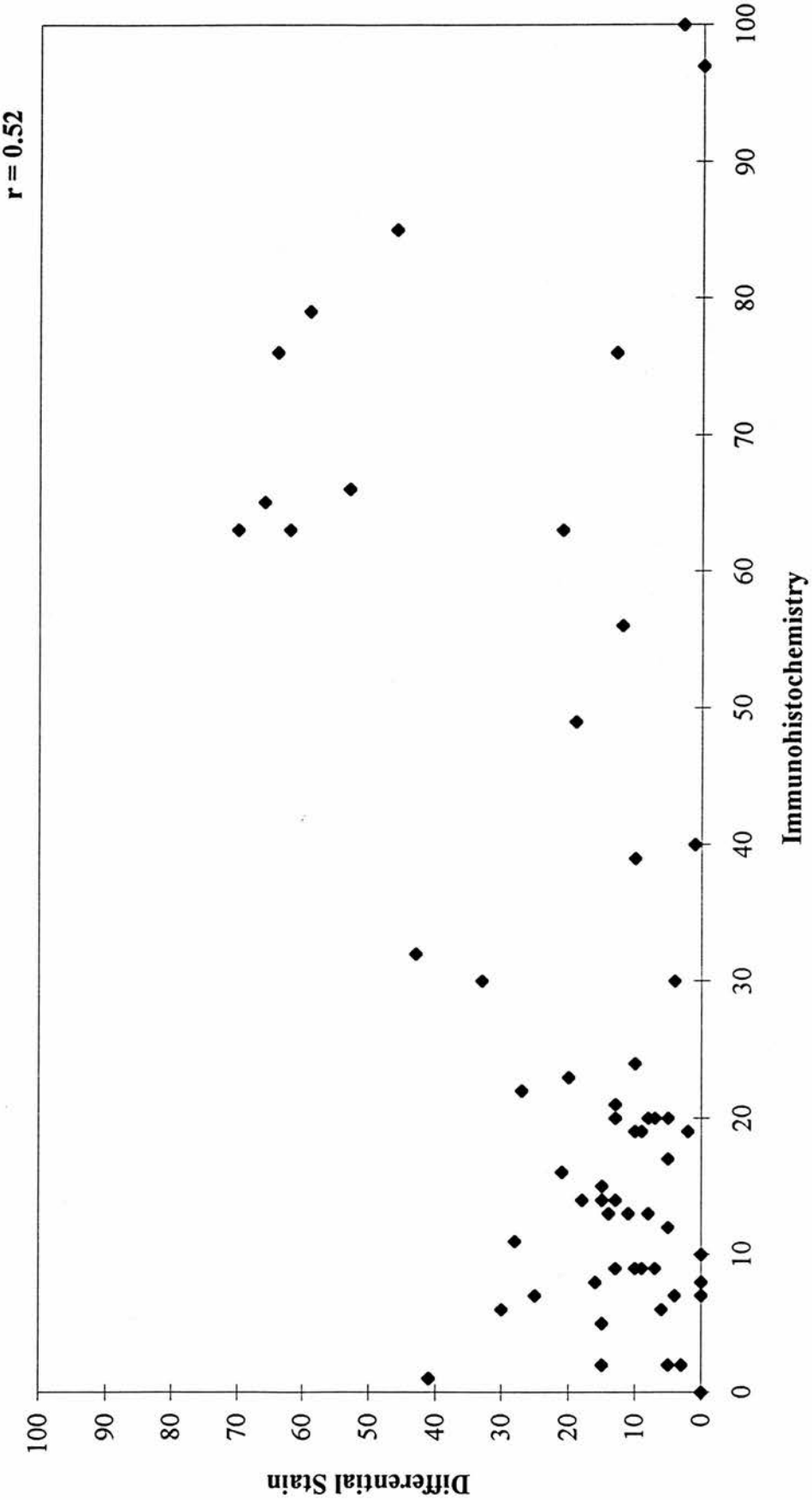
Photo 6.7: Anti-PMN x 400 oil immersion..



Graph 6.1: Scatter Plot for Neutrophils



Graph 6.1: Values are percentage of 300 cells counted that stained positively for neutrophils



Graph 6.2: Values are percentage of 300 cells counted that stained positively for macrophages

6.5.3 Discussion

The neutrophils methods agree well with only a few results being completely in disagreement. On the whole the correlation was good.

The differential stain and CD-68 stain do not appear to be measuring the same cell types and the differential stain underestimates the amount of monocytes/macrophages present. There is a cluster of samples at approximately 60% where both methods agree. Perhaps a clear abundance of macrophages makes them easy to recognise morphologically. There are slides where the immunohistochemical stain would appear to underestimate the number of macrophages compared to the differential stain.

6.5.4 Conclusion

The neutrophils are easily identified by differential stain but macrophage numbers are under-estimated. The use of immunohistochemical stains in the studies of bronchoalveolar lavage cell populations may be more accurate.

7. Cell Culture Model of Inflammation

7.1 Introduction

The study of the inflammatory process in the lungs of preterm neonates is hampered by many factors. Whilst it is relatively easy to sample lung effluent from intubated neonates, the samples vary greatly in size and current methods do not allow bronchoalveolar lavage samples to be adjusted for dilutions factors. This makes comparison between studies difficult. We can only sample from infants that are intubated and as infants in whom respiratory problems resolve are extubated, we do not have an adequate control group for comparison. Infants that are intubated receive many different clinical treatments e.g. surfactants, steroids; some of which may have a direct bearing on the lung inflammatory response. Infants may also be infected with *U. urealyticum* or colonised with other organisms. As clinical practices between neonatal units vary it is almost impossible to produce two infants with exactly the same clinical course and outcome. It would be useful to elaborate the lung inflammatory response under more controlled conditions.

7.1.1 Animal Models

Several investigators have used animal models in an attempt to standardise conditions and treatments that may lead to the development of chronic lung disease and (deLemos *et al.*,1992) recently reviewed the current animal models. Premature labour is rare in most species except man, however rabbits, sheep, rats, baboons and guinea pigs can all be electively delivered prematurely. Rabbits, rats and sheep cannot be maintained successfully for prolonged periods but provide models of respiratory distress syndrome. Newborn guinea pigs have relatively mature lungs compared to humans with events such as differentiation and alveolarisation which occur pre-dominantly postnatally in humans occurring prenatally in guinea pigs (Frank.,1983). Non-human primates provide the best model as they can be maintained for longer periods but generally recover without developing chronic lung disease.

7.1.2 Cell Based Models

A cell based model has many advantages over animal models. It is cheap, requires little specialist equipment or staff and avoids any moral or ethical considerations. There are many cell types present in the lung which are involved in the inflammatory response. The alveolar macrophage is the most important as it regulates the response. However fibroblasts, epithelial cells and endothelial cells which greatly outnumber macrophages have a very important role to play in the migration of neutrophils into the lungs, a central event in the inflammatory process. They express adhesion molecules which facilitate the migration of neutrophils and are capable of IL-8 production.

A recent study by Stancombe *et al* 1993 investigated neonatal pulmonary fibroblasts from primary cell culture and used *U. urealyticum* to stimulate cytokine production. Their results showed that *U. urealyticum* was stimulatory and induced the production of IL-8 and IL-6 in a dose dependent manner.

Cells from a primary culture of premature neonatal lungs would be the ideal for an assay, however this is not practical. It would require repeated sampling from infants or the transformation of the neonatal cells. There are commercially available human cell lines which are immortalised but their transformation may cause their responses to differ from any *in vivo* responses. Their advantages are that they are relatively easy to keep and obviate the need for continual sampling of neonates. Our model uses a human lung epithelial cell line (A549).

7.1.3 A549 Cells

A549 (ATCC CCL-185) cells are human lung carcinoma cells which are epithelial-like. They can synthesise lecithin using the cytidine diphosphocholine pathway and have multilamellar inclusion bodies. These are characteristics of type II lung epithelial cells, but they can not be definitively characterised as such.

Studies using A549 cells have shown that these cells can be stimulated by TNF-α, IL-α/β, viruses and bacteria to express IL-8, IL-6, RANTES, eotaxin and ICAM-1 (Kwon *et al.*,1994; Smart *et al.*,1994; Bittleman *et al.*,1995; Palfreyman *et al.*,1997; Arnold *et al.*,1996; Burkegaffney *et al.*,1996; Lilly *et al.*,1997; Arnold *et al.*,1994;

Kwon *et al.*,1995; Mizuno *et al.*,1994). They do not express TNF-α (Arnold *et al.*,1994) or MIP-1α (Kwon *et al.*,1995). The kinetics of their IL-8 response is different to primary cultures (Kwon *et al.*,1994). Primary cultures produce a peak of IL-8 at 2 hours and then a steady increase in IL-8 from about 8 hours. This second increase is probably due to *de novo* protein synthesis stimulated by another factor released from A549 cells in an autocrine manner. This second factor has not been elucidated. A549 cells produce a peak of IL-8 at about 4 hours with no secondary increase. The IL-8 produced from the A549 cells is much more immunoreactive than from primary cultures which suggests that primary cells have a regulatory mechanism which dampens IL-8 effects. Both these differences are likely related to the transformation of the A549 cells.

7.1.4 Culture of Ureaplasma urealyticum

U. urealyticum culture is carried out in growth medium supplemented with urea which is essential for the organism to grow. Urease enzymes breakdown urea which causes the pH to rise, and a colour change from orange to red. If dilutions of samples are incubated then the titre of bacteria present can be calculated from the lowest concentration of bacteria that causes a colour change. *U. urealyticum* has no cell wall and is sensitive to pH. The rising pH or red colour, which confirms bacterial growth in the medium, also kills the bacteria. *U. urealyticum* is a surface parasite, attaching via adhesins (Henrich *et al.*,1993) and the cell model requires live organisms. We therefore need to count the organisms in culture before the medium turns red. *U. urealyticum* is not visible using conventional light microscopy. The most accurate method of counting *U. urealyticum* is to count colonies grown in solid phase culture which are 15-30μm in diameter. A drop of liquid containing *U. urealyticum* can be cultured on solid A7 agar and incubated anaerobically for 48 hours. The colonies grown are black due to manganese sulphate which oxidises in the presence of *U. urealyticum.* Colonies can be counted on an inverted microscope and the titre of the specimen then calculated. This method takes 48 hours in which it is impossible to hold the culture in stasis. The culture must be used in the stimulation experiments "blind" i.e. without knowing the exact count of *U. urealyticum* until after the experiment is

finished. By using freeze-dried vials supplied from the Public Health Laboratories there is a good chance that reconstitution will result in counts of around 10^5 colony forming units per ml which based on other results (Stancombe *et al.*,1993) should be adequate for our experiments.

The aim of the following experiments was to further investigate the production of IL-8 from lung epithelial cells. The role of *U. urealyticum* and another genital isolate of *E. coli* on IL-8 production was investigated. The high oxygen concentrations an infant is subjected to by ventilation is thought to increase the level of oxygen radicals (Zimmerman.,1995; review) which are thought to cause tissue damage. Premature infants have reduced anti-oxidant enzyme defences so this tissue damage goes unchecked. Experiments investigating the stimulation of A549 cells cultured in a high concentration of oxygen were included to assess its effects on epithelial cells. In clinical practice infants receive surfactants, antibiotics and steroids and as these may have a direct effect on the IL-8 produced from lung epithelial cells these were also investigated.

7.2 Cell Culture Methods

7.2.1 Materials

75 cm^3 flasks (Costar, Cat No. 3376)

PBS (Life Technologies, Cat. No. 14190094)

Trypsin-EDTA (Life Technologies, Cat. No. 45300019)

DMEM (Life Technologies, Cat. No. 22320-022)

Fetal bovine serum (FBS) (Life Technologies, Cat. No. 10108-074)

L-glutamine (Life Technologies, Cat. No. 25030-032)

Penicillin/streptomycin (Life Technologies, Cat. No. 15070-022)

Dimethyl Sulphoxide (DMSO) (Sigma, Cat. No. D-2650)

Growth Medium (GM)

DMEM + 10% FBS + 2mM L-glutamine + 100U penicillin/streptomycin

Cell Freezing Medium (CFM)

DMEM + 10% FBS + 2mM L-glutamine + 100U penicillin/streptomycin + 1% DMSO

7.2.2 A549 Cell Line

All cell culture was carried out in a class II hood under sterile aseptic conditions. The cells were grown in an Heraeus incubator at 37°C with humidity and 5% CO_2. A549 cells were a gift from the Rayne Laboratory, Edinburgh University and were supplied as a living culture. The cells were cultured in 12.5 mls growth medium (GM) in 75 cm^3 flasks and split when confluent, approximately twice weekly. When confluence was reached the GM was removed and replaced with 12.5 mls sterile PBS and washed for 2 minutes. This was discarded and the cells were incubated with 5mls trypsin-EDTA at 37°C until the cells had become dislodged. 20 mls of GM was added to inactivate the trypsin. The resulting mixture was poured into a labelled 50 ml centrifuge tube and spun at 200g (Sigma 6K10) for 10 mins at room temperature. The supernatant was discarded and the cell pellet resuspended by first gently flicking the bottom of the tube and then adding 5mls GM. This wash step was repeated twice. 1ml was added to the new flasks containing 12.5 mls GM. These were incubated at 37°C until confluent and the process repeated.

7.2.3 Storing Cells in Liquid Nitrogen

For long term storage the cells must be kept in liquid nitrogen. Cells were trypsinised and spun down as described above. They were counted and resuspended in freezing medium (See "Appendix A p176) to a cell suspension of 2 x 10^6 - 8 x 10^6 cells/ml. They were then aliquoted into 1ml cryotubes and frozen at -70°C overnight. The following morning they were transferred to liquid nitrogen and stored until revival.

Revival was performed by pre-warming GM to 37°C. The cryotube was removed from liquid nitrogen and the temperature rapidly raised to 37°C in a water bath. Once

the pellet could be dislodged from the cryotube it was transferred to a flask containing 12.5 mls of pre-warmed GM and left overnight at 37°C, 5% CO_2. The next day the medium was removed and replaced with fresh GM and the cells maintained as before.

7.3 Bacterial Culture Methods

7.3.1 Materials

Nutrient Broth (NB) (South East Blood Transfusion Centre, Edinburgh)
Bacterial Freezing Medium (BFM) - Nutrient broth + 10% glycerol
Urea-arginine LYO broth (UAL) (bioMérieux, France Cat. No. 42 504)
A7 solid agar plate (bioMérieux, France Cat. No. 43 003)

7.3.2 Eschericia coli Culture

A clinical genital isolate of *Eschericia coli* (*E. coli*) was streaked onto a blood agar plate. It was inoculated into 200 mls NB and incubated for 24 hours at 37°C with constant shaking. The culture was then spun at 3000g for 5 mins and the supernatant discarded. The pellet was resuspended in 20ml freezing medium and the culture was checked for purity both by Gram-stain (See "Section 7.3.3: Gram Stain for Purity") and blood agar (See "Section 7.3.4 Blood Agar Test for Purity"). The bacterial cell concentration was counted and adjusted to 1 x 10^{10} cells/ml and 1ml aliquots were frozen at - 70°C until use. Viability was assessed weekly to ensure the cultures had survived freezing.

7.3.3 Gram Stain for Purity

A 5-10µl drop of culture was placed onto a glass slide and allowed to air dry before being passed through a naked flame. The slide was flooded with methyl violet for 1-2 mins and rinsed off with tap water. The slide was flooded with iodine for 1-2 minutes and the excess drained off. The slide was flooded with acetone for 10 seconds and washed immediately with tap water. Finally, the slide was flooded with basic fuschin for 30 seconds, rinsed in tap water and blotted dry. The slide was viewed under oil

immersion at x400 magnification. Red cells are Gram positive, purple are Gram negative.

7.3.4 Blood Agar Test for Purity

A sterile metal loop was used to place a small drop of culture onto a blood agar plate. The loop was flamed and then used to streak the culture in three straight lines across the plate. The loop was flamed again and at the end of those three lines streak three more straight lines were streaked across the plate. This process was repeated once more until the plate boundary was completely covered in streaks. The plate was incubated overnight at 37°C and checked for purity.

7.3.5 Viability Count

Four concentrations of each culture were made in NB. Five 10µl aliquots of each dilution were then dropped onto blood agar plates and allowed to air dry before being transferred to a 37°C incubator. They were incubated overnight and the colonies in each drop were counted and averaged over the five aliquots.

7.3.6 urealyticum Culture

U. urealyticum was obtained as a freeze dried sterile culture from the NCTC (National Collections of Type Cultures, The Public Health Laboratory Service, UK, Cat. No. 10177). It was reconstituted in 3 mls of UAL and the colony forming units counted (See below). However, as this process takes 48 hours to gain an accurate count the reconstituted culture was used in the stimulation assay, and a retrospective count of colony forming units was taken.

Colony Forming Units (CFUs)

To estimate the CFU 3 x 20µl of reconstituted freeze dried vial was cultured on solid A7 agar and incubated anaerobically for 48 hours. Anaerobic environment was generated using CampyPak™ Microaerophilic system (Becton Dickinson) which produces an atmosphere of 5 to 12% carbon dioxide and a residual atmosphere of 5 to 15% oxygen.

The colonies are black due to manganese sulphate which oxidises in the presence of *U. urealyticum.* Five random fields of view were counted in each of the 20µl drop using an inverted microscope at x100 and the counts averaged. The titre of the specimen is then calculated using the formulae supplied by bioMérieux shown in Table 7.1.

Table 7-1: Calculation of U. urealyticum Titre

Colonies per field	Titre of specimen (CFU)
< 1	10^3
1 - 5	10^4
5 - 15	10^5
> 15	10^6

7.4 Interleukin-8 Measurements

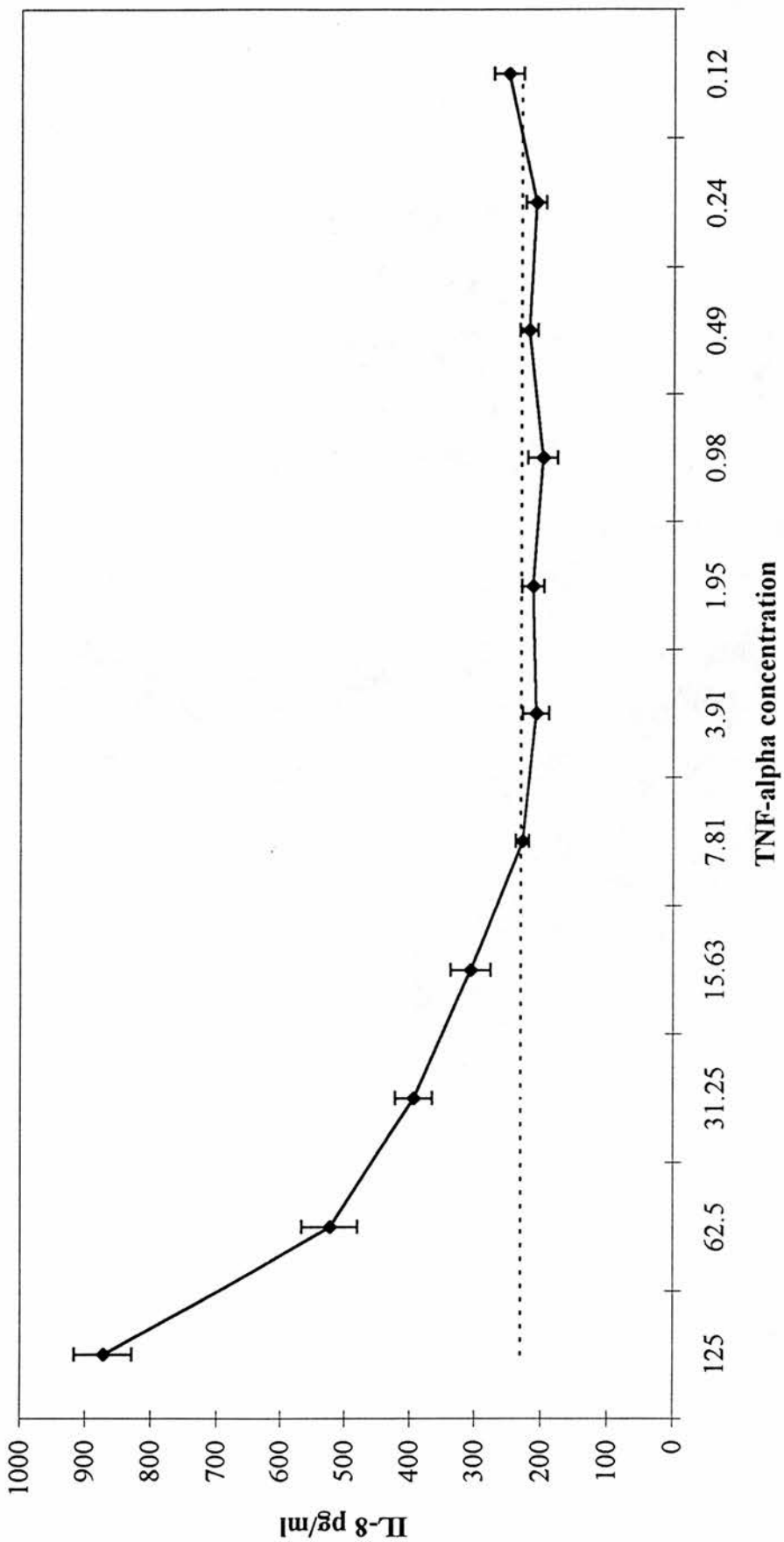
This was the radioimmunoassay as described in Chapter 3 p60. Samples were tested in triplicates.

7.5 Assay Constituents Concentrations

7.5.1 Tumour Necrosis Factor - α

A549 cells were plated out as per 7.6.2 in a 96 well plate at 2 x 10^5 cells/ml. TNF-α (NIBSC, batch 87/650) was diluted in AsM from 2500 pg/ml to 0.15 pg/ml (100 IU/ml to 0.006 IU/ml). 50µl of each dilution was incubated triplicate wells and the resultant supernatants assayed for IL-8 production. Wells containing no TNF-α were also included in the assay.

Graph 7.1: TNF-alpha Stimulation Assay



Graph 7.1: Values are means, error bars are 95% confidence intervals. Dotted line is the mean value when no TNF-alpha is added to the cells.

Results and Discussion

The raw data are given in Table 10-20, Appendix B. The first four dilutions of TNF-α were above the highest value on the standard curve and so were not plotted on Graph 7.1. All concentrations of TNF-α above 7.81pg/ml gave a significantly increased IL-8 response from the A549 cells (students T test). To increase the IL-8 response marginally and not maximally TNF-α was used at 32 pg/ml (1.28 IU/ml) in stimulation assay, which gives approximately a doubling of the IL-8 response from the cells alone.

7.5.2 Antibiotics and Dexamethasone

It is difficult to establish an *in vitro* dose to use that is relative to the clinical dose. It was therefore decided to use a high, medium and low dose of these constituents, namely 10μg/ml, 1μg/ml and 100ng/ml, with 50 μl added to each well.

7.5.3 Surfactants

Exosurf and Curosurf were too expensive to buy and our pharmacy had none spare. On administration to an infant there is usually 1-2 mls left in a vial and this was collected over successive days, pooled and stored at 4°C until use. Both surfactants are reconstituted to different amounts of the active constituent dipalmitoylphospatidylcholine (DPPC); Curosurf has 80 mg/ml and Exosurf has 13.5 mg/ml. However they are administered differently and it was decided to keep DPPC levels of each of the surfactants the same. As high concentrations of Exosurf can be toxic to cells (Findlay *et al.*,1995) both surfactants were used at 500ng/ml, 50ng/ml and 5ng/ml DPPC, with 50 μl added to each well.

7.5.4 Bacterial Cell Concentrations

E. coli was used at three concentrations 1×10^8, 1×10^6 and 1×10^4 E. coli/ml diluted in AsM. *U. urealyticum* was more difficult to calculate due to the retrospective nature of the CFU count. In general reconstituting a vial of freeze-dried *U. urealyticum* resulted in about 10^6 CFU/ml. This was diluted in 20 mls of AsM to give roughly a final concentration of 5×10^5 CFU/ml in 3 mls UAL. This was further diluted to 5 x

10^4 and 5 x 10^3 in assay medium (see below). Each assay with *U. urealyticum* gives the exact CFU count.

7.6 Stimulation Assay Development

7.6.1 Materials

Gentamicin (Sigma, Cat. No. G1272)

Erythromycin (Sigma, Cat. No. E-5389)

Dexamethasone (Sigma, Cat. No. D2915)

Curosurf (Serono, Italy)

Exosurf (Wellcome Burroughs)

96-well culture plates (Costar, Cat. No. 3599)

Assay Medium (AsM)

DMEM + 10% FBS + 2mM L-glutamine

Fetal Bovine Serum

Fetal bovine serum (FBS) contains proteins and growth factors essential for cell growth in culture. It may also stimulate IL-8 production from A549 cells. At the beginning of the assay development several samples of batches were obtained from Life Technologies and were incubated with the A549 cells for 24 hours. The IL-8 levels were compared to unstimulated cells and a batch of FBS which produced no increase in IL-8 was used throughout all of the following experiments.

7.6.2 Plating Cells onto Microtitre Plates

The cell monolayer in each well should be ~ 90% confluent and 2 x 10^5 cells/ml was found to be that on visual examination of several experiments (results not shown).

The cells were trypsinised as before and washed three times in DMEM before being resuspended into 5 mls GM. The cell number was counted in a haemocytometer and then adjusted to 2 x 10^5 cells/ml. 100µl was added to each well of a 96-well culture plate and the cells were left to adhere overnight at 37°C. The GM was removed by

aspiration and the cells were washed in 100µl/well of PBS which was aspirated before stimulation.

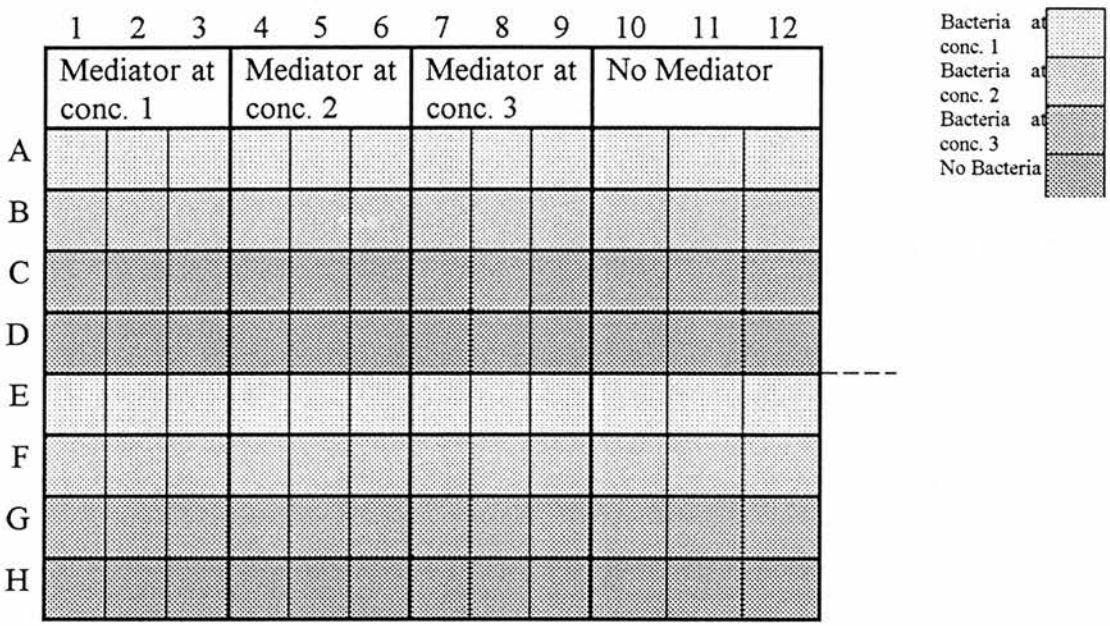
7.6.3 Microtitre Plate Layout

All plates were laid out in the same way as shown in

Figure 7-1. All constituents were added at 50µl per well. Wells were made up to a final volume of 200µl using AsM and the plates were incubated for 24 hours either in an oxygen enriched atmosphere as described in

Figure 7-2 or at 37°C with 5% CO_2 in an Heraeus incubator.

Figure 7-1: Plate layout for all constituents in the stimulation assays.



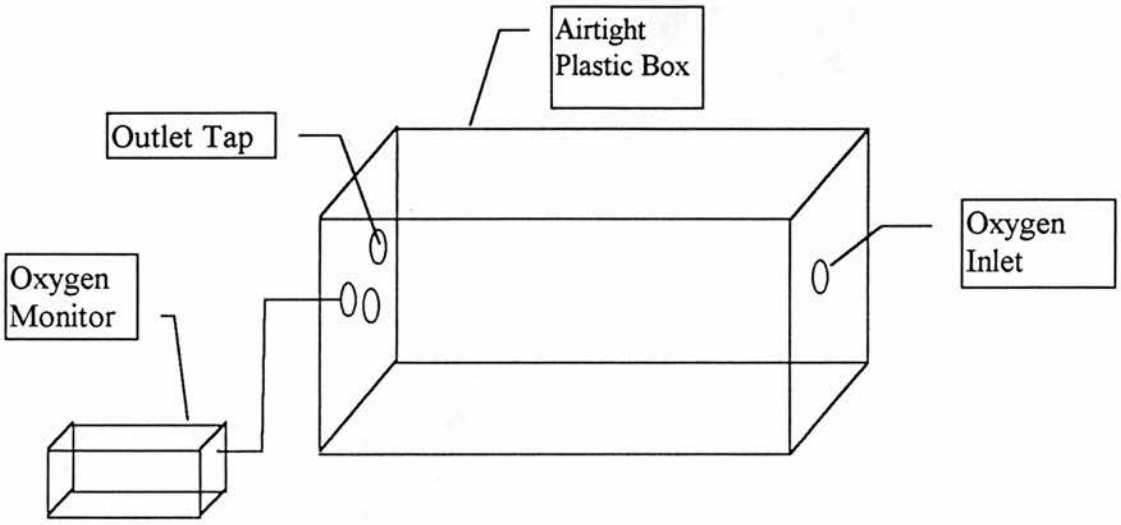
7.6.4 Oxygen Enriched Atmosphere

To generate an oxygen enriched atmosphere an airtight box was connected to an inlet and outlet through which oxygen gas could be passed (

Figure 7-2). The box was filled with oxygen and once the oxygen monitor read 80% the box was sealed and incubated for 24 hours at 37°C. The oxygen levels in the box

were measured after 24 hours incubation and were generally between 30 - 40 % which is significantly above room air concentration.

Figure 7-2: Schematic of the box used to provide an oxygen enriched atmosphere in which to incubate microtitre plates.



7.7 Stimulation Assays

For each bacterium three stimulation assays were performed using identical plate layouts. Each bacterium was therefore stimulating A549 cells in the presence or absence of TNF-α; with three different concentrations of antibiotic, surfactant or steroid; in CO_2 or an oxygen enriched environment.

Each bacterium with one of the mediators was set up exactly the same on six plates on one day; three plates were incubated in the Heraeus incubator at 37°C with 5% CO_2 and three plates were incubated at 37°C in the oxygen enriched atmosphere. All six plates were incubated for 24 hours. 100µl was removed from each well taking care not to dislodge any cells on the bottom of the microtitre plate and frozen at -20°C until IL-8 assay.

7.8 Statistical Analysis

The IL-8 values were averaged for the three plates and a multi-factor analysis of variance was performed on the logarithms of the IL-8 values. The main factors were TNF-α, oxygen, bacterial cell concentration and mediator concentration and these were tested for main effects. Two-way and three-way interactions between the main factors were also explored. Further analyses were t-tests.

The p values for the ANOVA are given in Table 7.2 for U. urealyticum and in Table 7.3 for E. coli. Graphs for all the data follow the results tables and results and discussion starts on page158.

Table 7.2: ANOVA Significance values for Ureaplasma urealyticum

The presence or absence of TNF-α and oxygen; or the concentration of mediators. Also reported are two and three way interactions between all components in the assay.

	Main Effect				Two Way Interaction						Three Way Interaction			
Mediators	**TNF**	**O_2**	**Uu**	**Med**	**TNF O_2**	**TNF Uu**	**TNF Uu**	**O_2 Uu**	**O_2 Med**	**Uu Med**	**TNF O_2 Uu**	**TNF O_2 Uu**	**TNF Uu Med**	**O_2 Uu Med**
Erythromycin	*<.001*	*<.001*	.402	*.01*	.158	.637	.112	.317	.189	.838	.112	.332	.667	.500
Gentamicin	*<.001*	*<.001*	.209	*.005*	*.014*	.681	.088	.348	*.006*	.976	.284	*.016*	.91	.593
Exosurf	*.001*	*<.001*	*<.001*	*<.001*	.106	*<.001*	.123	.871	*.025*	.158	.334	.631	.174	.459
Curosurf	*.026*	*<.001*	*<.001*	*<.001*	*.005*	*.004*	.429	.566	.569	.248	.118	.198	.211	.308
Dexamethasone	*<.001*	*<.001*	*<.001*	*<.001*	.952	.266	.124	.234	*<.001*	.691	.29	.806	.949	.734

Abbreviations: TNF = TNF-α ; O_2 = oxygen; Uu = U. urealyticum; Med = mediator (as shown in the first column).

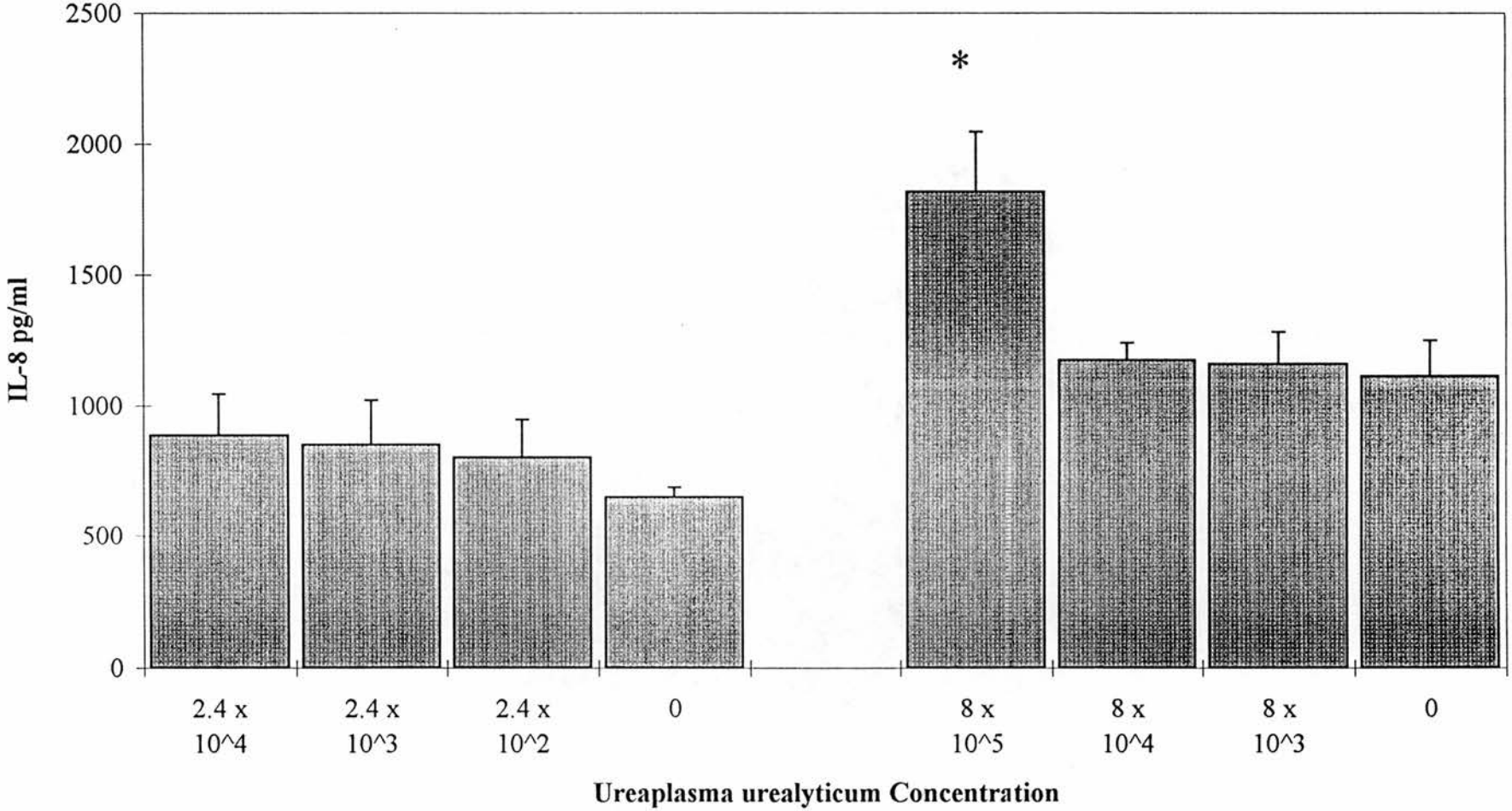
Table 7.3: ANOVA Significance values for E. coli

The presence or absence of TNF-α and oxygen; or the concentration of mediators. Also reported are two and three way interactions between all components in the assay.

	Main Effect				Two Way Interaction						Three Way Interaction			
Mediators	**TNF**	**O_2**	**Ec**	**Med**	**TNF O_2**	**TNF Ec**	**TNF Ec**	**O_2 Ec**	**O_2 Med**	**Ec Med**	**TNF O_2 Ec**	**TNF O_2 Ec**	**TNF Ec Med**	**O_2 Ec Med**
Erythromycin	*.002*	*.003*	*<.001*	.112	.763	.952	.934	.239	.121	.659	.358	.617	.718	.846
Gentamicin	*<.001*	*<.001*	*<.001*	*.007*	.091	.449	.605	.752	.664	.248	.476	.754	.987	.772
Exosurf	*<.001*	*.001*	*<.001*	*.02*	.282	*.001*	.104	*.015*	.087	.088	.347	.391	.585	.194
Curosurf	*<.001*	*<.001*	*<.001*	*.03*	*.043*	*<.001*	.292	*<.001*	*.049*	.064	*.02*	.079	.37	.082
Dexamethasone	*<.001*	*<.001*	*<.001*	*<.001*	.334	.534	.685	.295	*.05*	*.002*	.183	.35	.939	.682

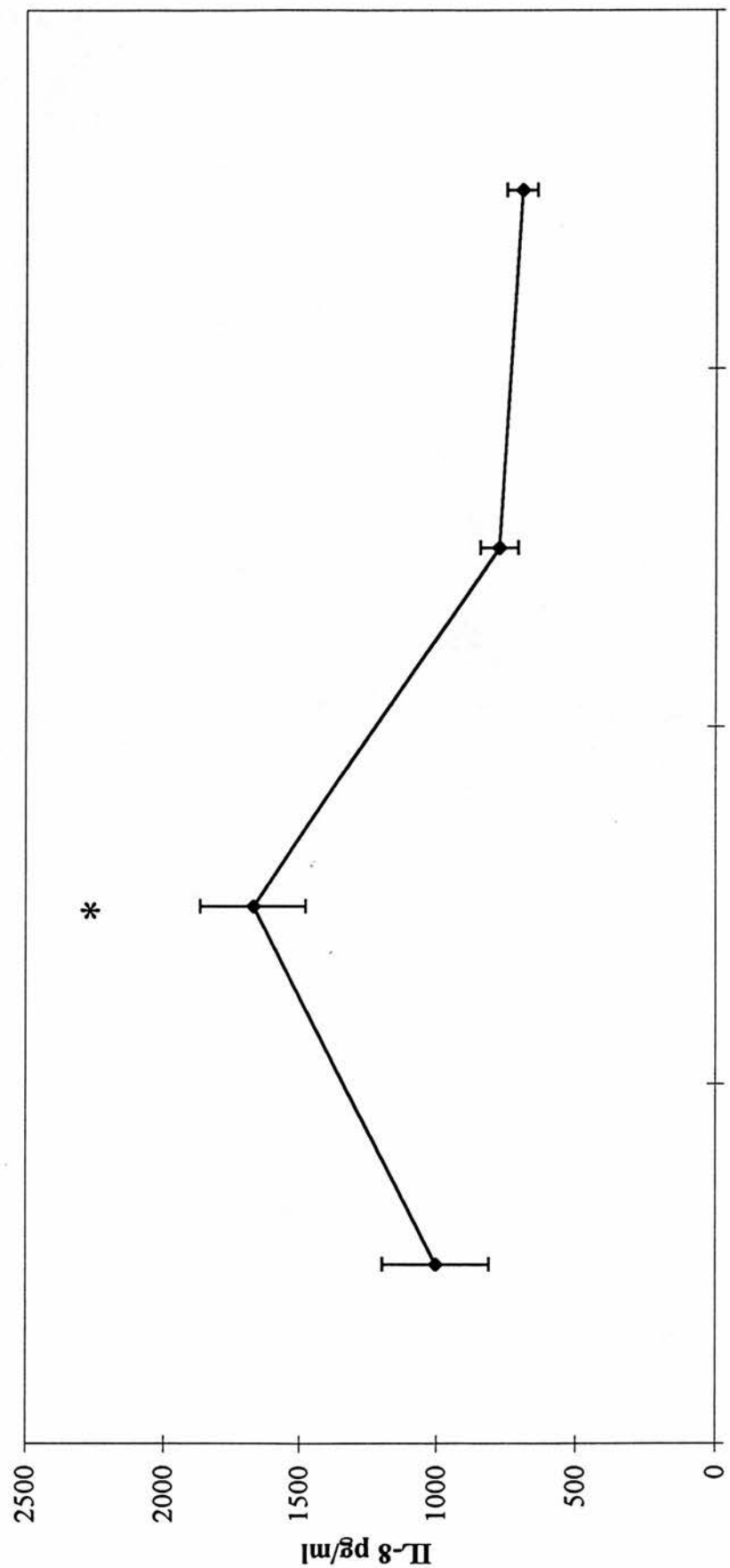
Abbreviations: TNF = TNF-α ; O_2 = oxygen; Ec = E. coli; Med = mediator (as shown in the first column).

Graph 7.2: Effect of U. urealyticum Concentration on IL-8 Production from A549 Cells



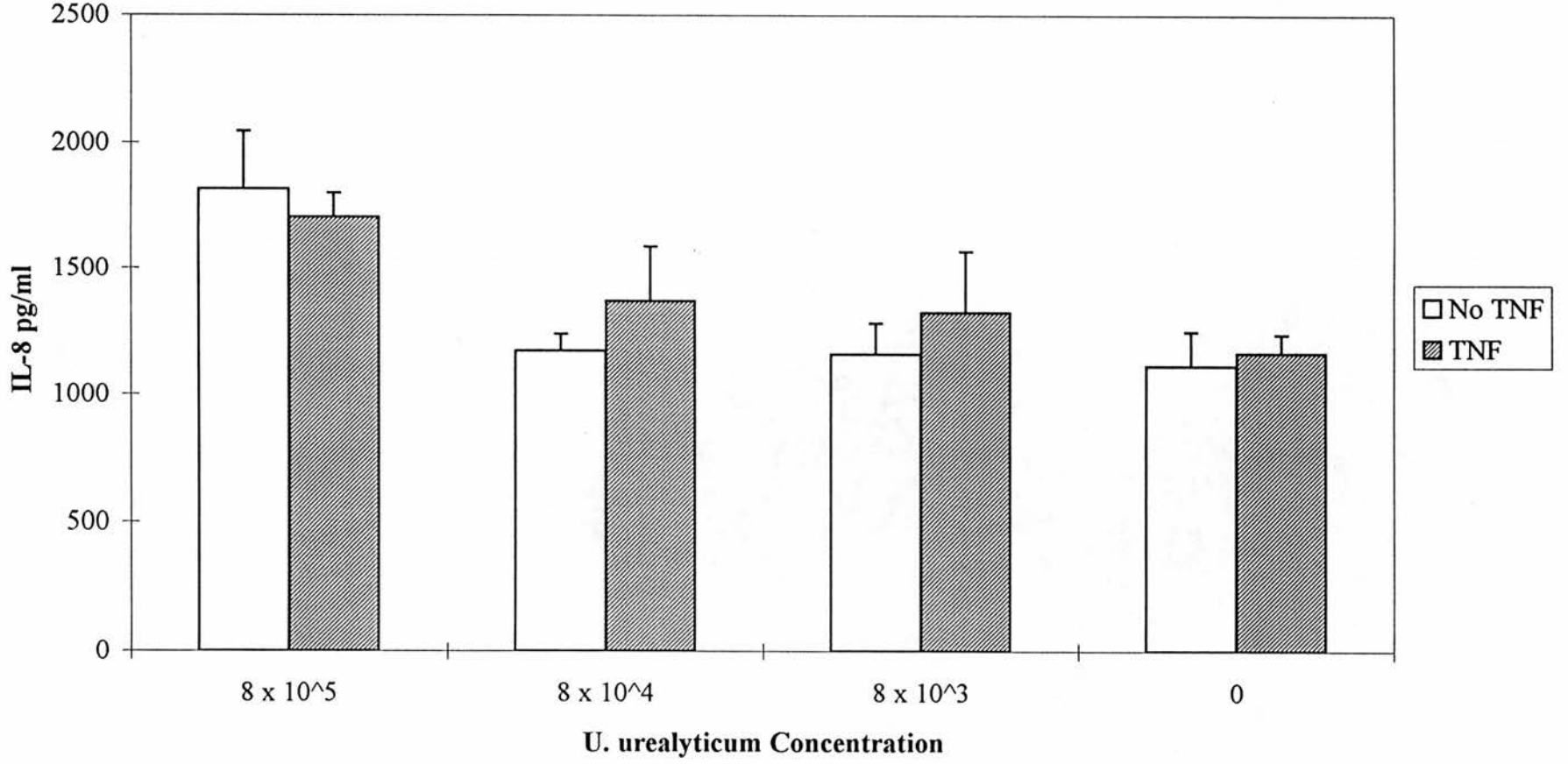
Graph 7.2: Values are means, error bars are standard error of the mean. * = $p < 0.03$ t-test compared to control.*

Graph 7.3: Effect of E.coli Concentration on IL-8 Production from A549 Cells

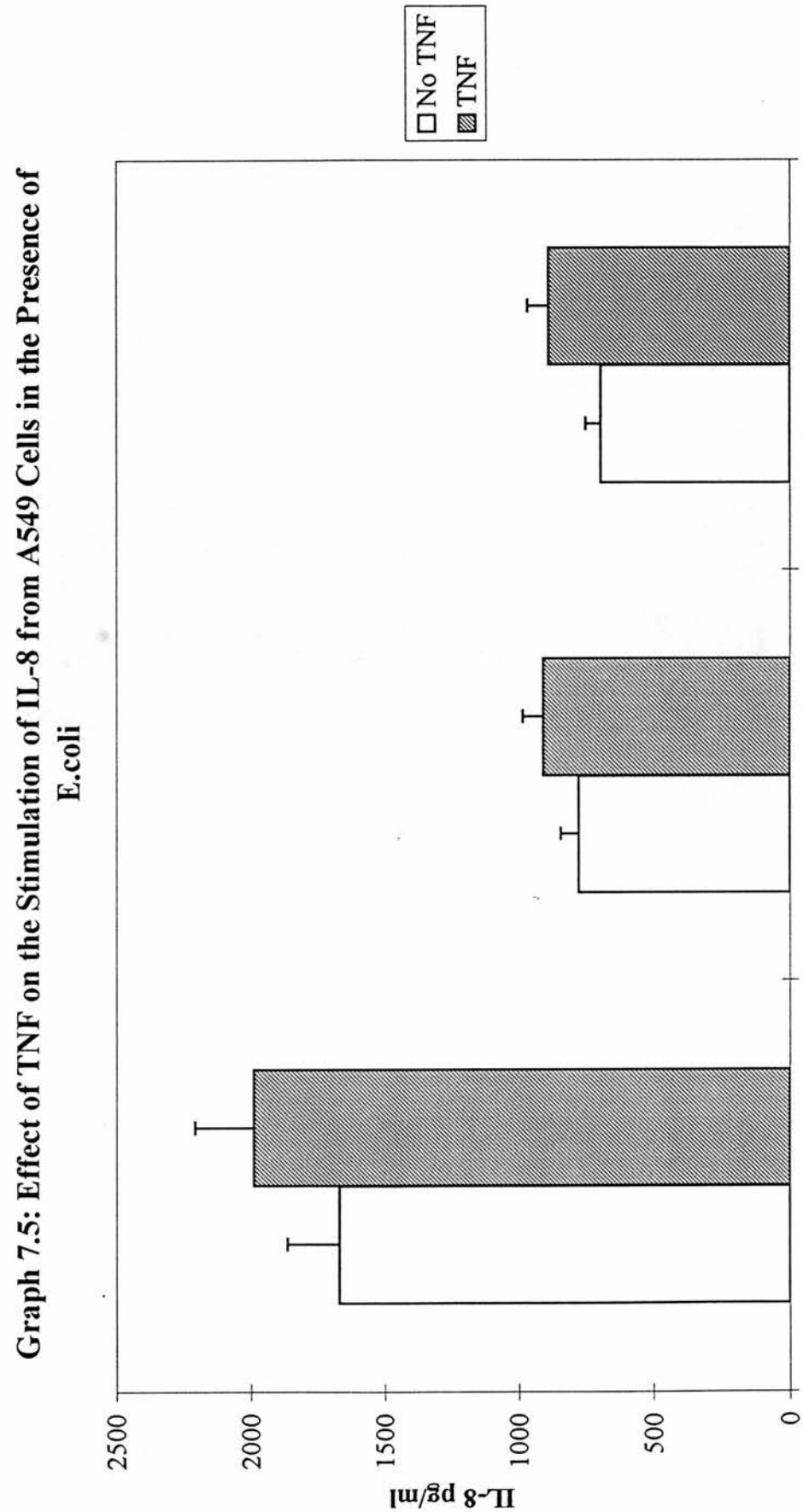


*Graph 7.3: Values are means, error bars are standard error of the mean. * = p = 0.0002 t-test compared to control.*

Graph 7.4: Effect of TNF on the Stimulation of A549 Cells in the Presence of U. urealyticum

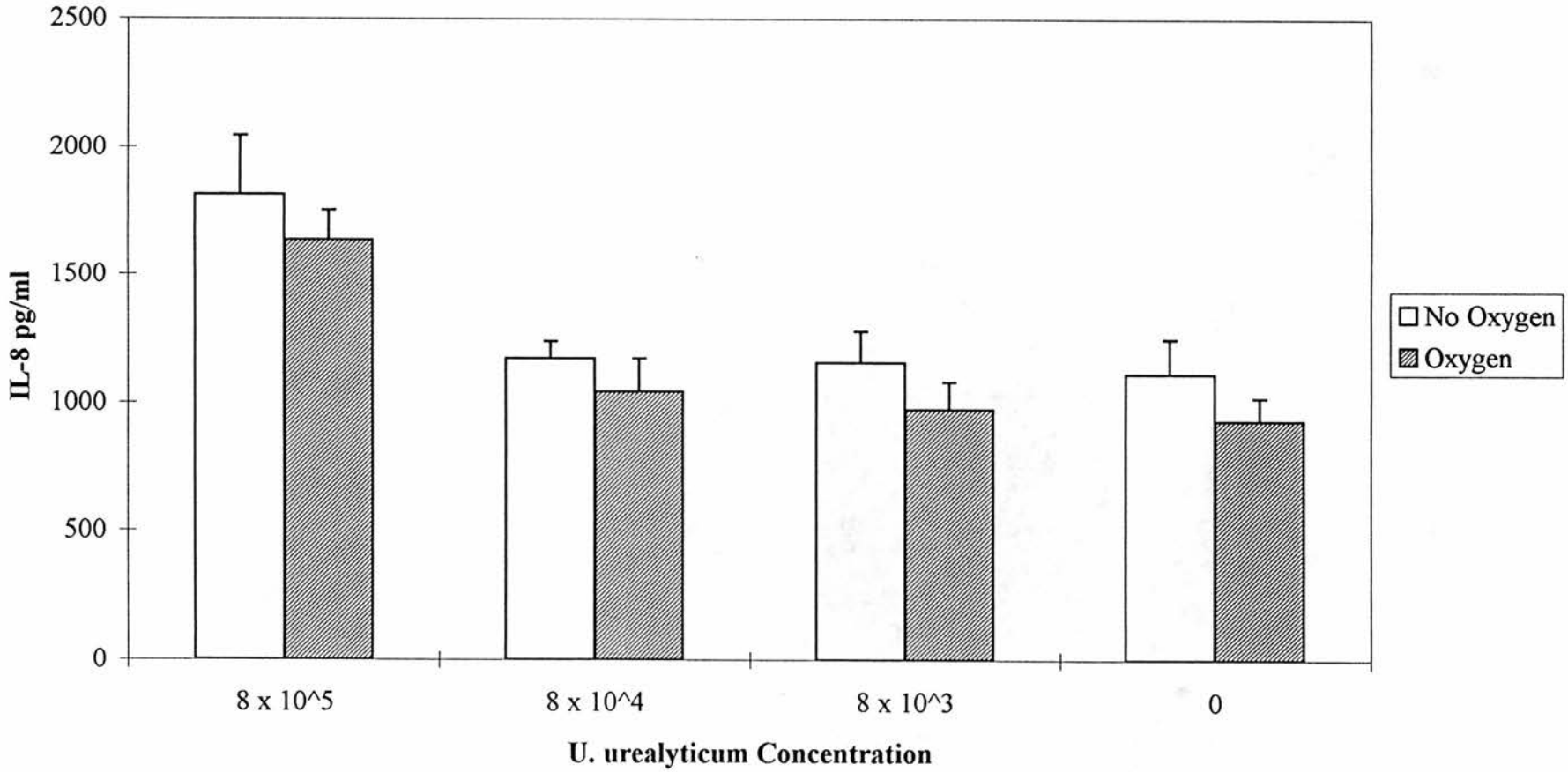


Graph 7.4: Values are means, error bars are standard error of the mean.



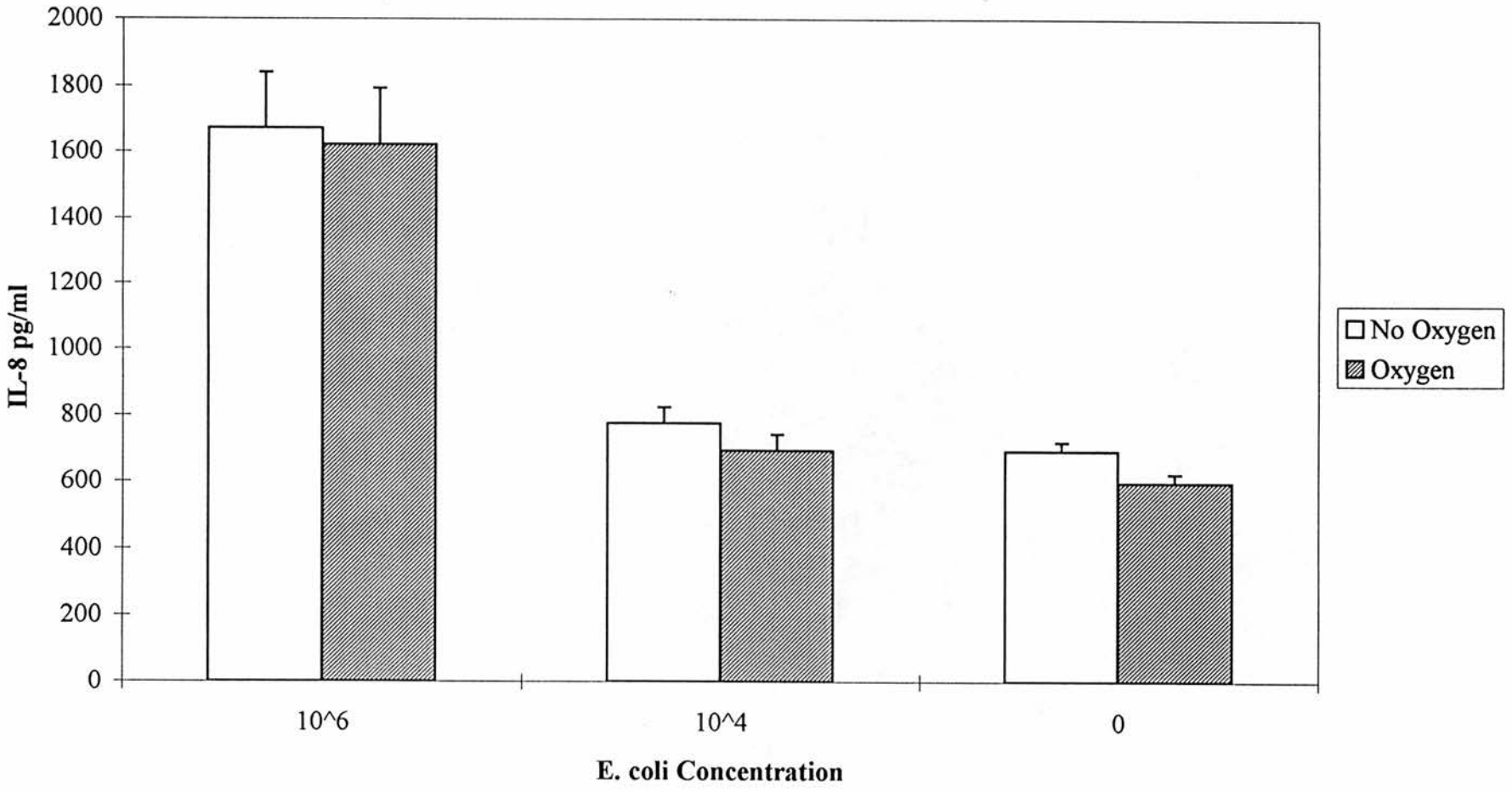
Graph 7.5: Values are means, error bars are standard error of the mean.

Graph 7.6 : Effect of Oxygen on the Stimulation of IL-8 from A549 Cells in the Presence of U. urealyticum



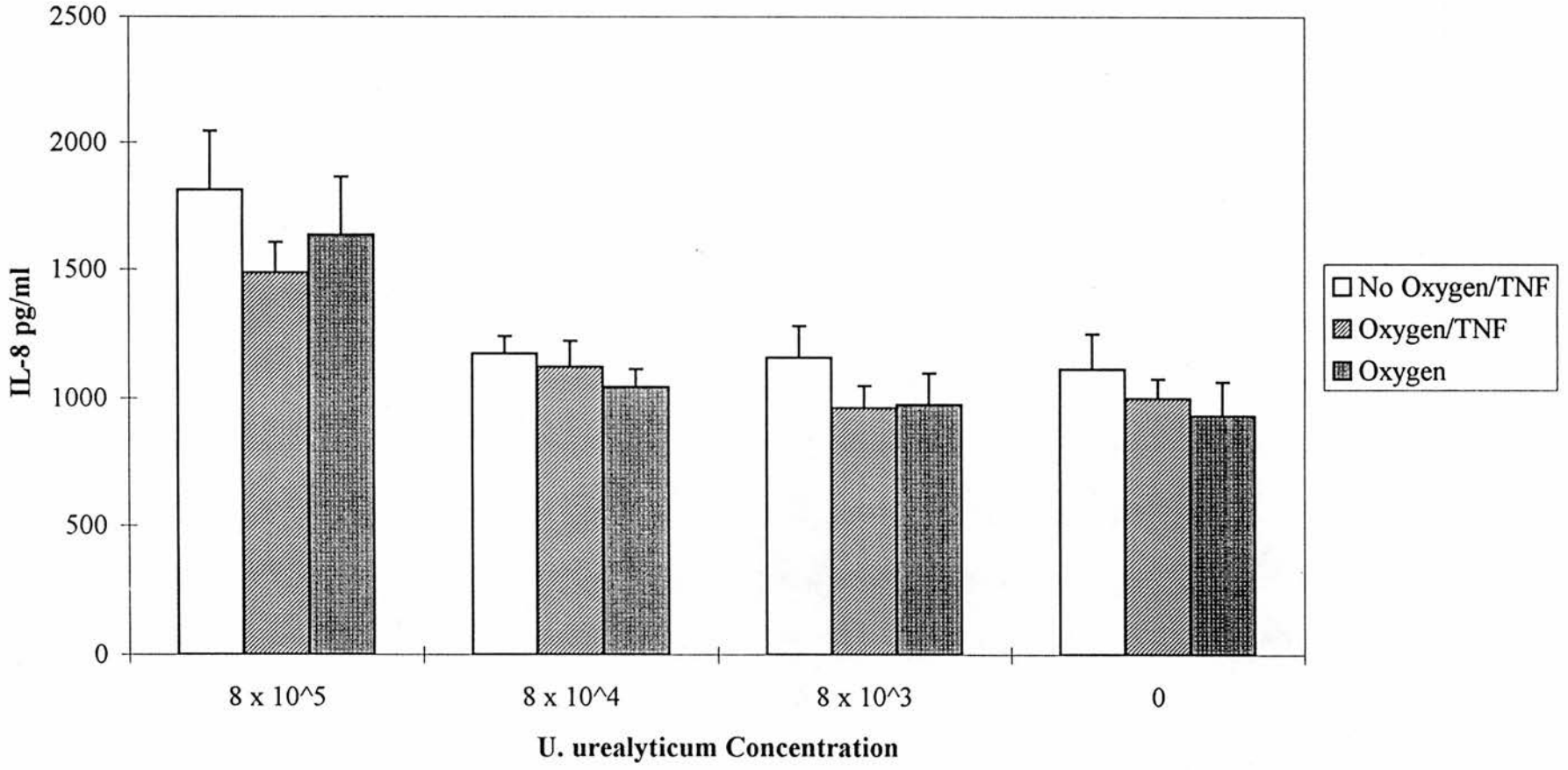
Graph 7.6: Values are means, error bars are standard error of the mean.

Graph 7.7 : Effect of Oxygen on the Stimulation of IL-8 from A549 Cells in the Presence of E. coli

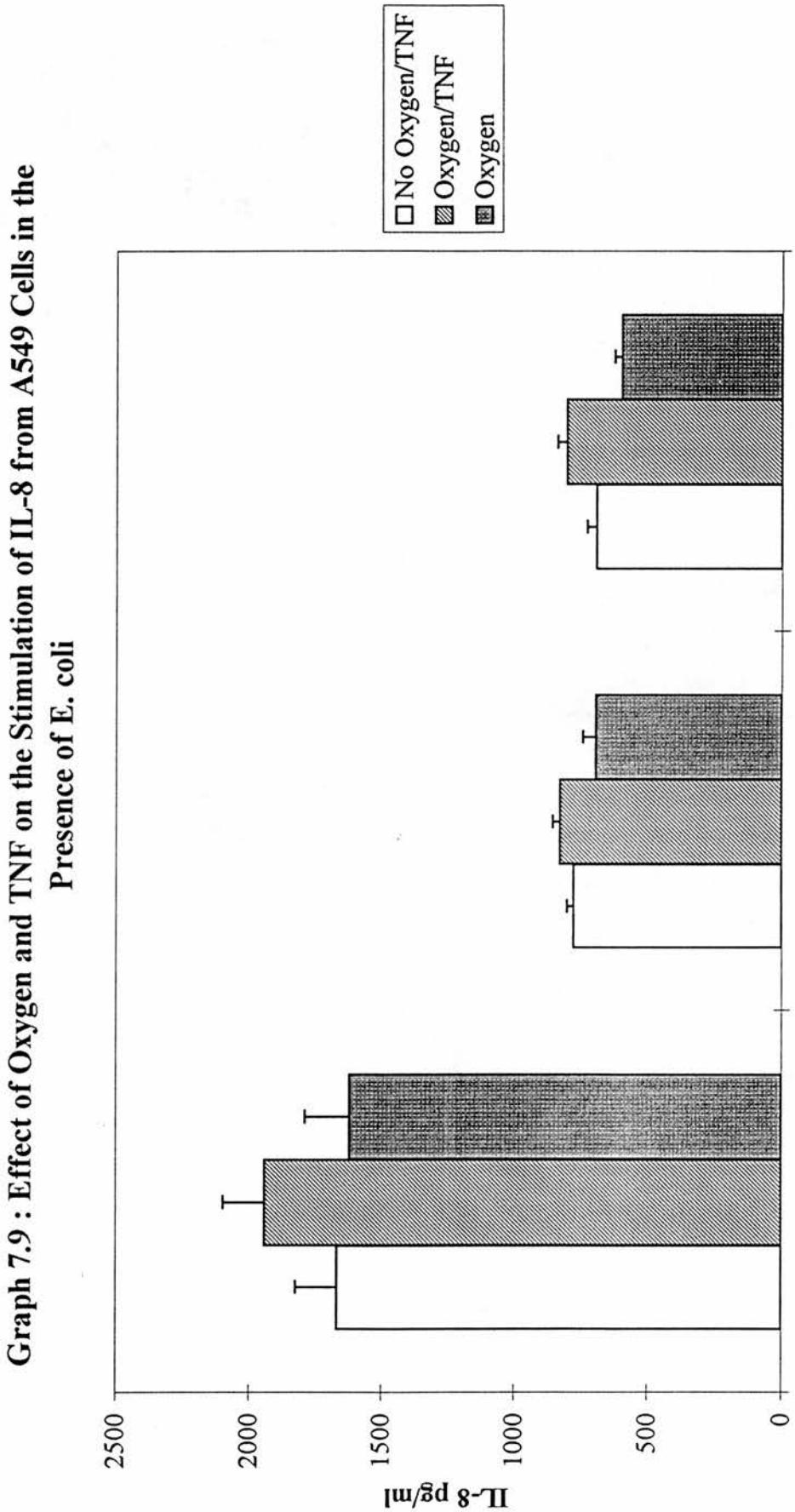


Graph 7.7: Values are means, error bars are standard error of the mean.

Graph 7.8 : Effect of Oxygen and TNF on the Stimulation of IL-8 from A549 Cells in the Presence of U. urealyticum

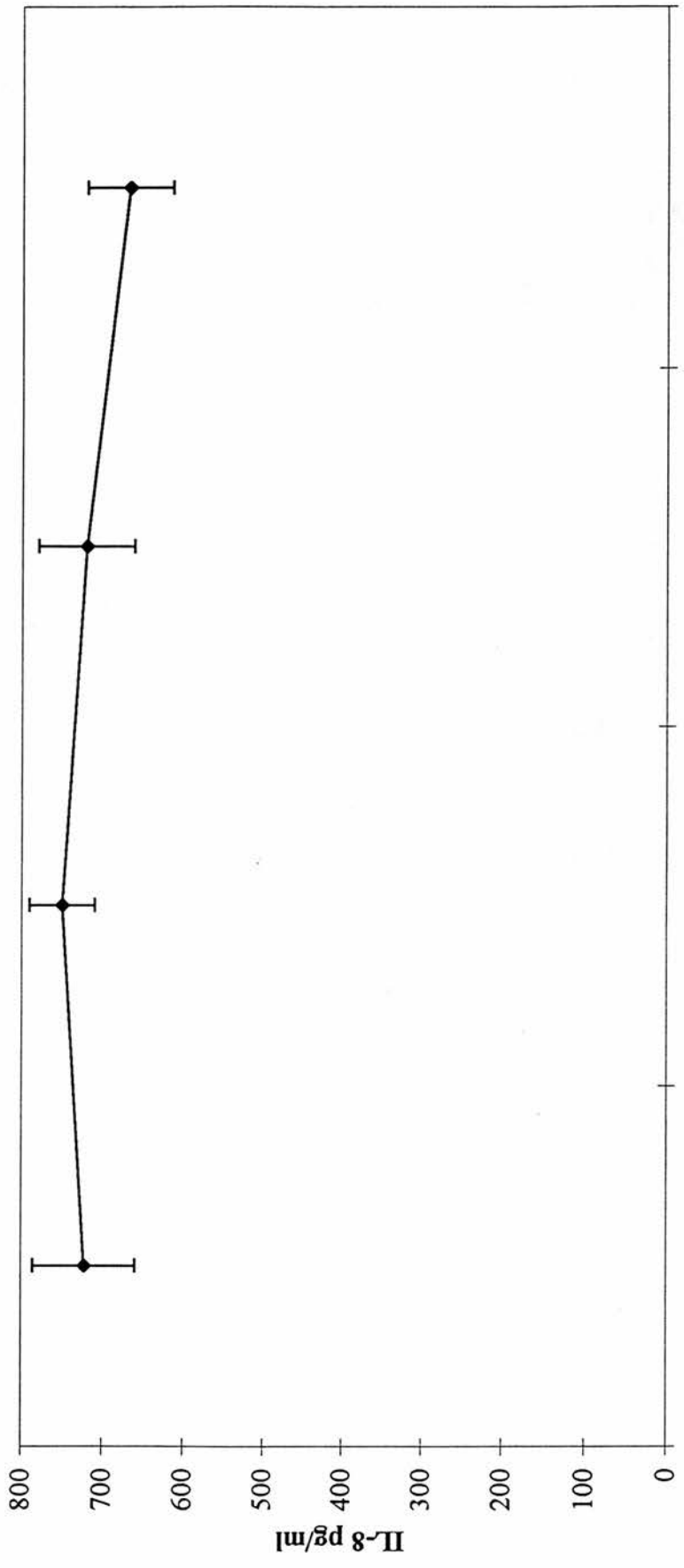


Graph 7.8: Values are means, error bars are standard error of the mean.



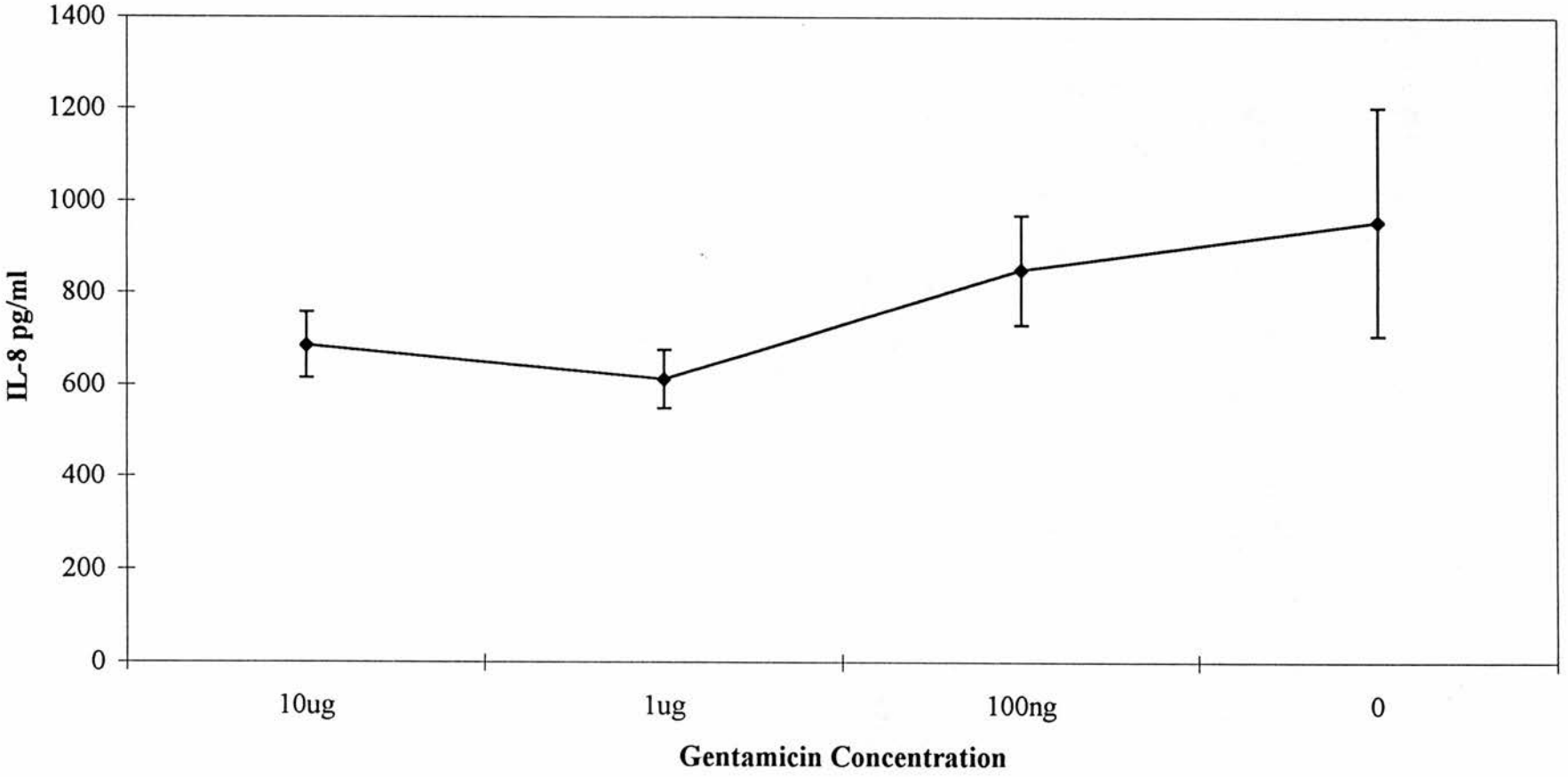
Graph 7.9: Values are means, error bars are standard error of the mean.

Graph 7.10 : Effect of Erythromycin on Production of IL-8 from A549 Cells in the Presence of U. urealyticum



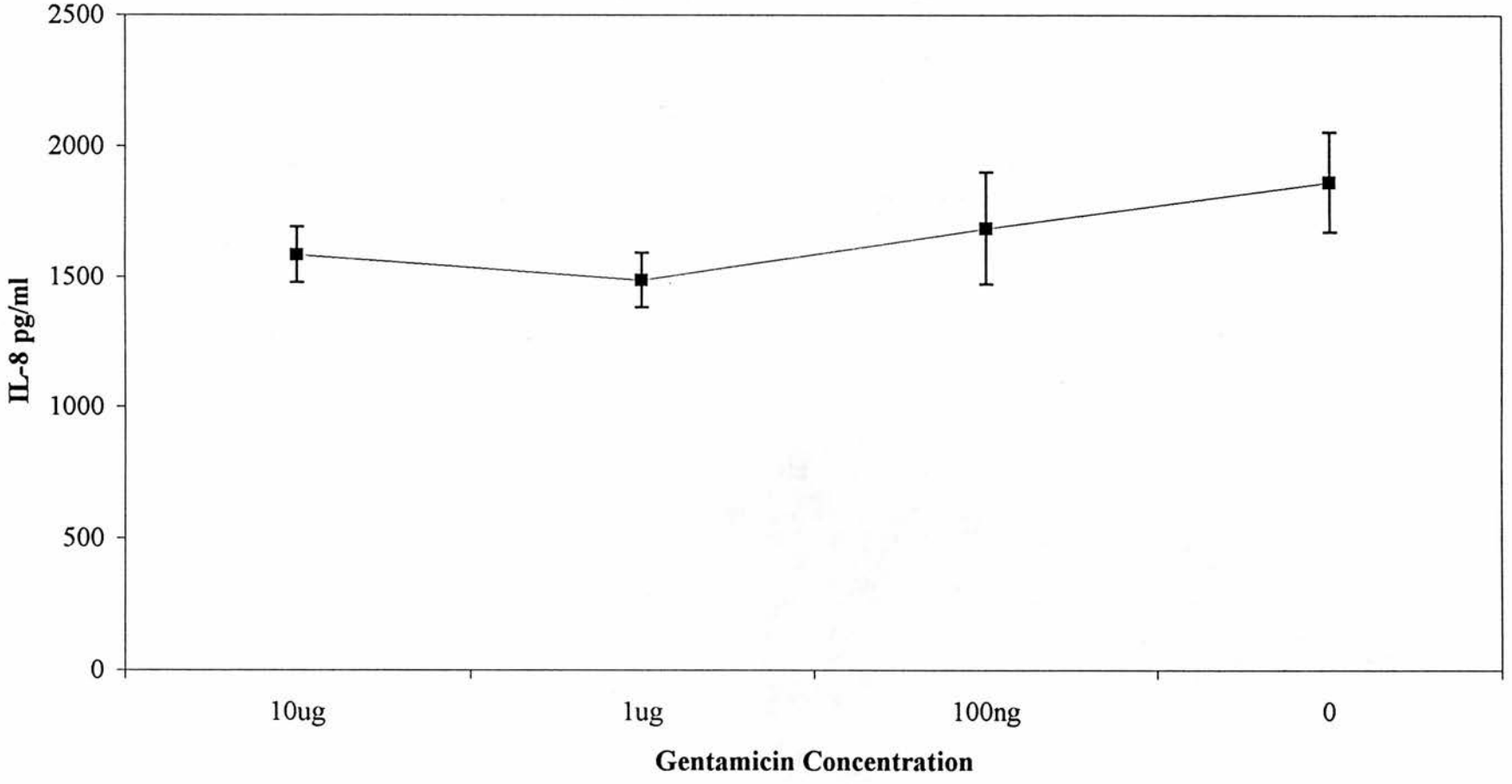
Graph 7.10: Values are means, error bars are standard error of the mean.

Graph 7.11 : Effect of Gentamicin on Production of IL-8 from A549 Cells in the Presence of U. urealyticum



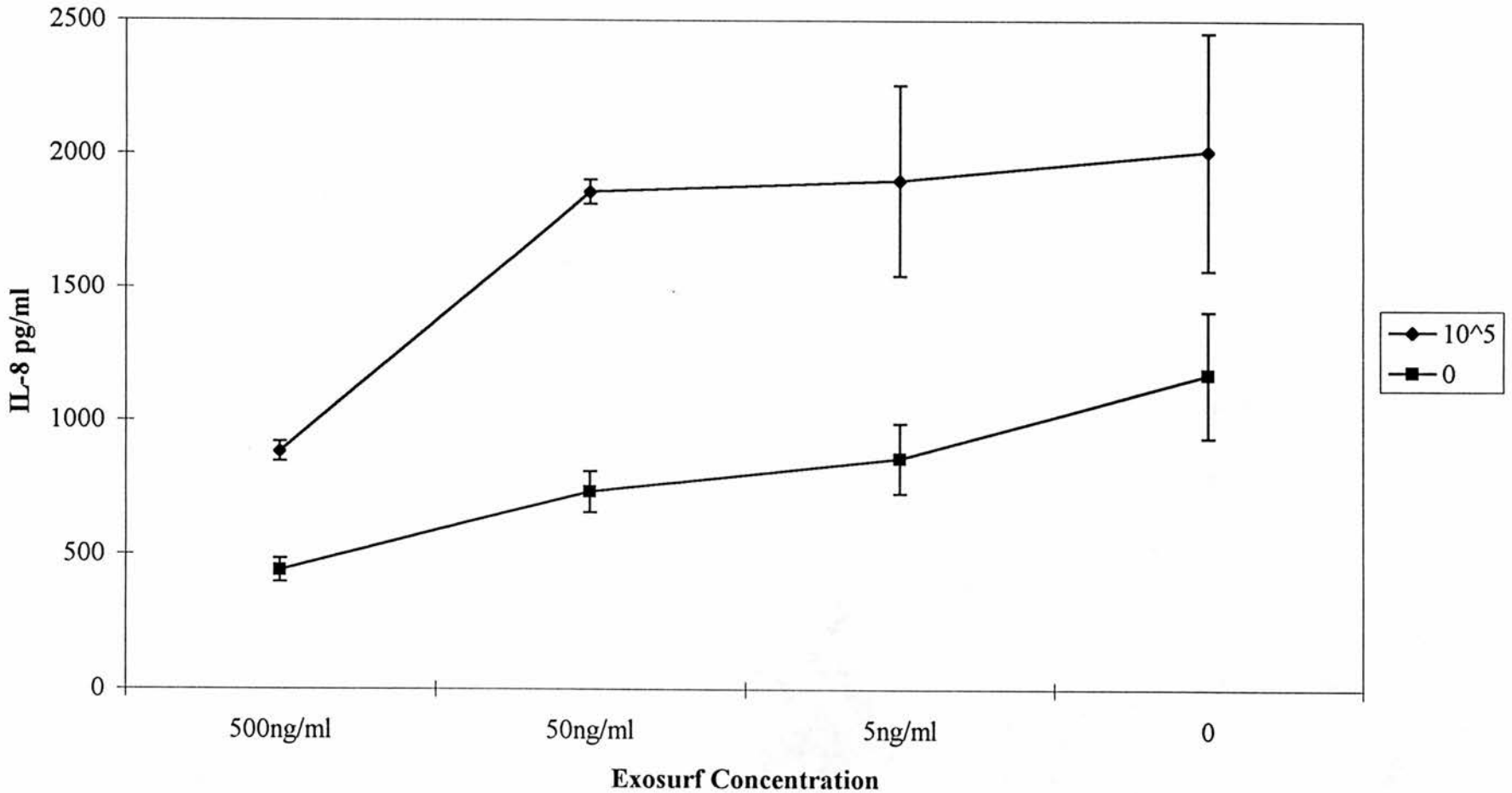
Graph 7.11: Values are means, error bars are standard error of the mean.

Graph 7.12: Effect of Gentamicin on Stimulation of IL-8 from A549 Cells by 10^6 E.coli/ml



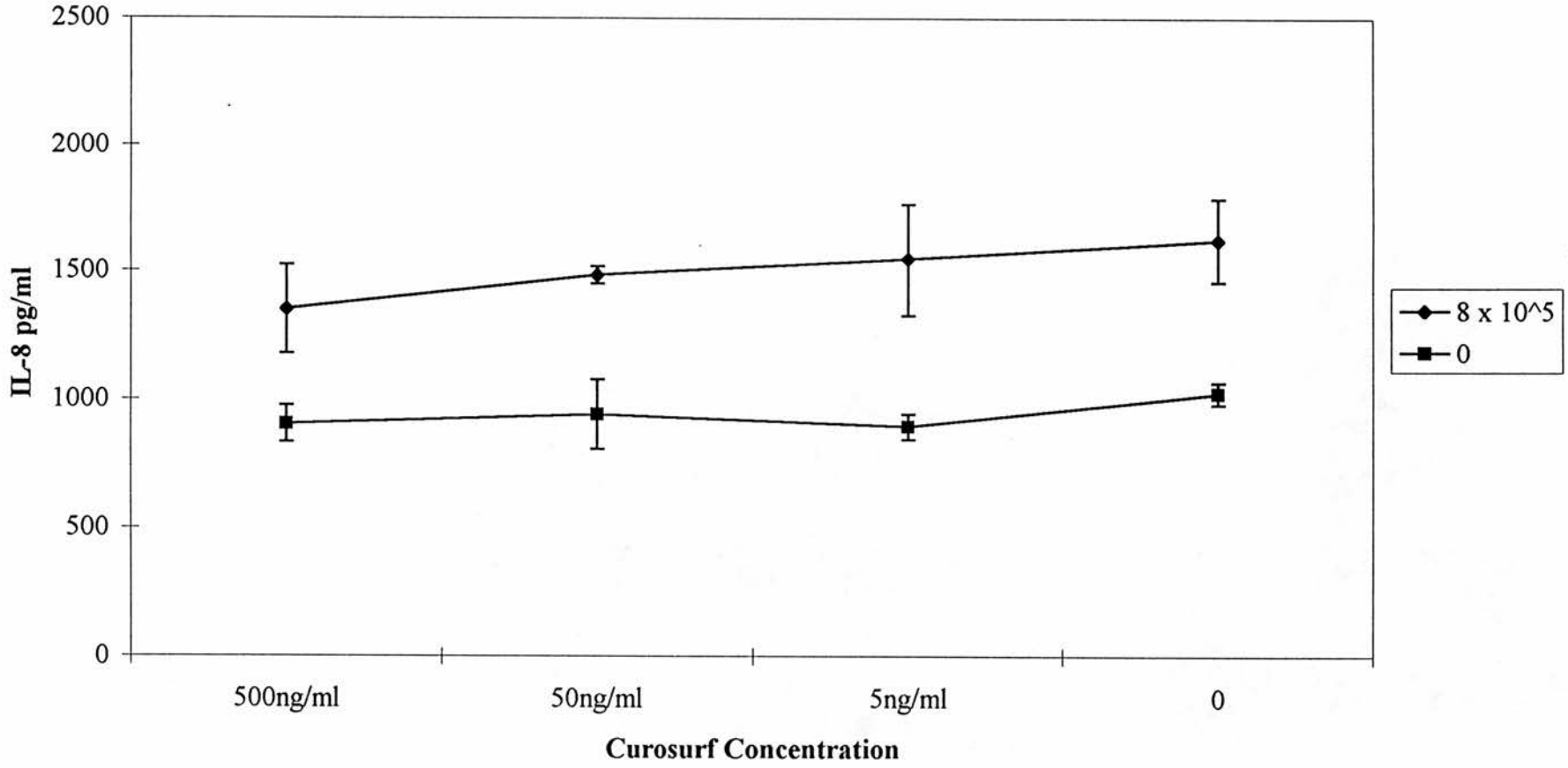
Graph 7.12: Values are means, error bars are standard error of the mean.

Graph 7.13: Effect of Exosurf on Stimulation of IL-8 by U. urealyticum



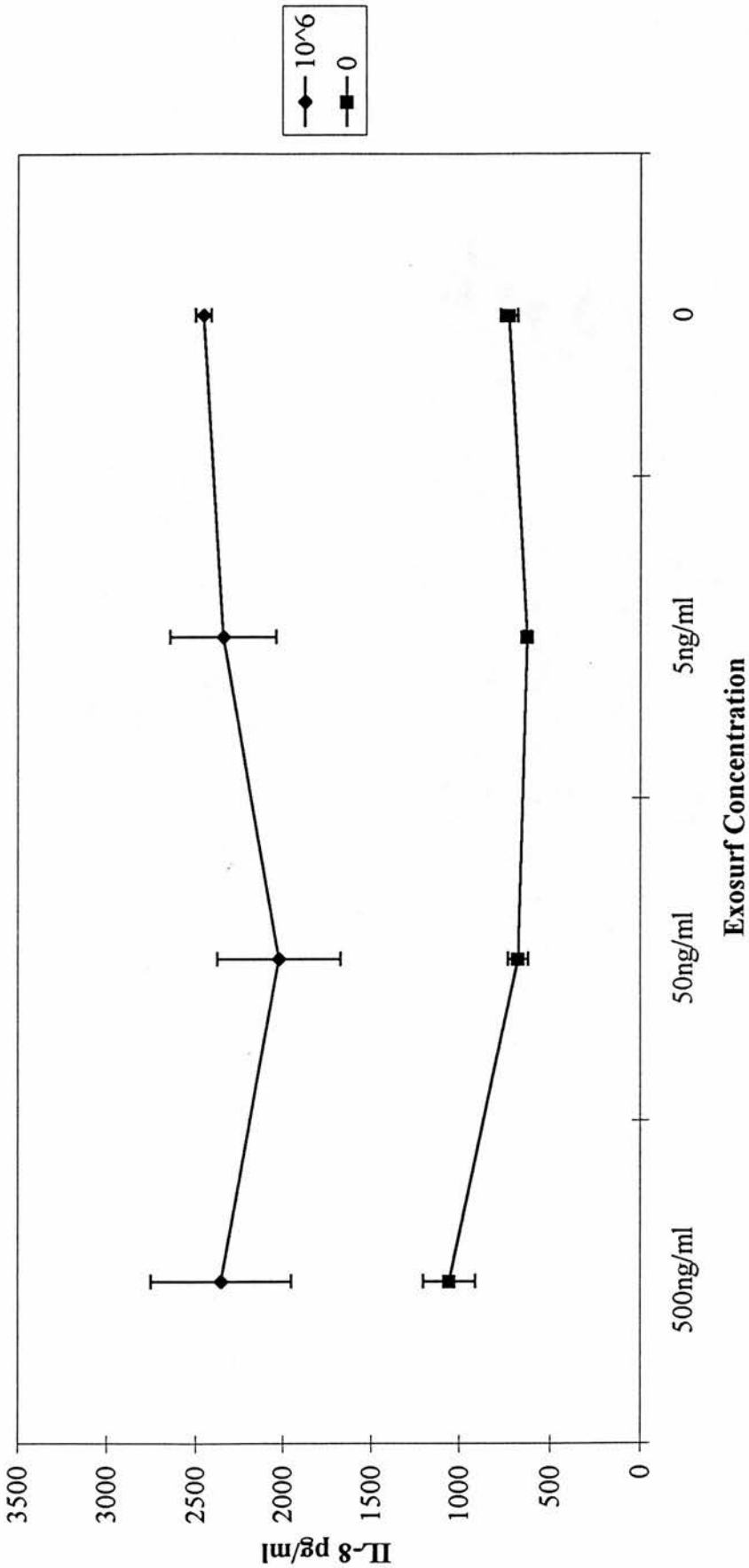
Graph 7.13: Values are means, error bars are standard error of the mean.

Graph 7.14: Effect of Curosurf on Stimulation of IL-8 by U. urealyticum



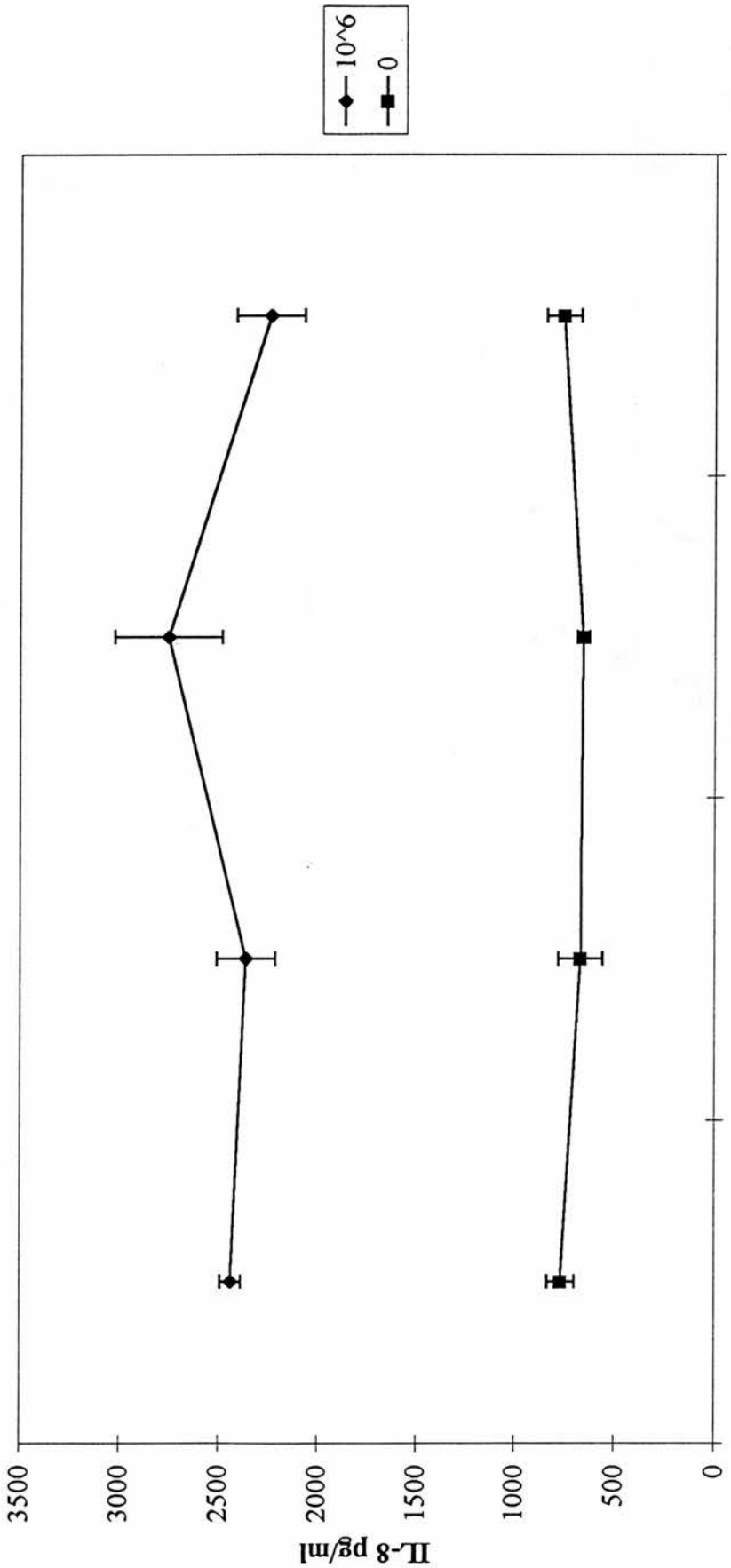
Graph 7.14: Values are means, error bars are standard error of the mean.

Graph 7.15: Effect of Exosurf on Stimulation of IL-8 by E. coli



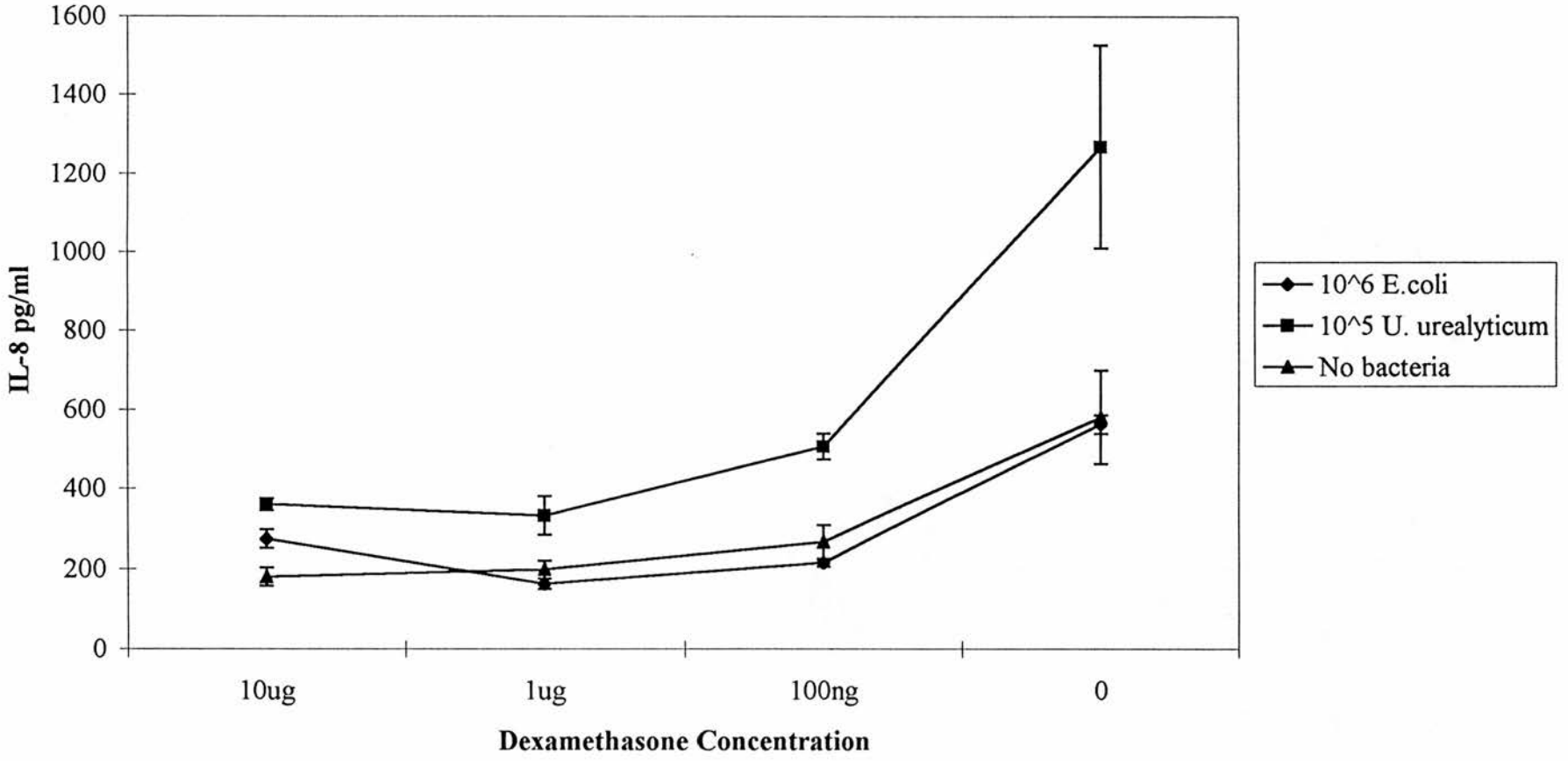
Graph 7.15: Values are means, error bars are standard error of the mean.

Graph 7.16: Effect of Curosurf on Stimulation of IL-8 by E.coli



Graph 7.16: Values are means, error bars are standard error of the mean.

Graph 7.17: Effect of Dexamethasone on Stimulation of IL-8 from A549 Cells in the Presence of U. urealyticum and E. coli



Graph 7.17: Values are means, error bars are standard error of the mean.

7.9 Results and Discussion

The problems of culture of *U. urealyticum* make this assay which requires live organisms difficult to set up. *U. urealyticum* is sensitive to pH due to its lack of a rigid cell wall. As a culture grows the pH of the culture medium rises which requires that cultures must be harvested before a fatal rise in pH has occurred. This timing is very difficult to establish and indeed there is considerable variation between cultures. The second problem is that these organisms are not visible using a light microscope due to their size. To estimate numbers they must cultured on solid agar and the colonies at 15 - 30μm can be counted using an inverted microscope. This process takes 48 hours during which time it is not possible to keep *U. urealyticum* cultures in stasis. The cultures must therefore be used without knowing the number of organisms present.

Three batches of *U. urealyticum* were grown U. urealyticum at different times and used to stimulate the A549 cells without knowing the number of CFU present. The first batch was used in the erythromycin and gentamicin experiments and yielded a concentration of 2.4×10^4 CFU/ml. The second batch used in the Curosurf/Exosurf experiments yielded 8×10^5 CFU/ml and the third batch used in the dexamethasone experiment yielded 1.5×10^5 CFU/ml. From Table 7.2 it is clear that *U. urealyticum* stimulates IL-8 production at $> 10^5$ CFU/ml as there was a significant main effect ($p<0.001$) for Exosurf, Curosurf and Dexamethasone, but not for erythromycin and gentamicin experiments. Graph 7.2 shows 10^5 CFU/ml produces an increase in IL-8 of 35% compared to when no *U. urealyticum* is present and this is significant at $p = 0.03$, t-test.
It was not possible to repeat the erythromycin and gentamicin experiments because of time constraints.

There was a significant main effect of E. coli concentration as shown in Table 7.3 in all experiments. *E. coli* can be frozen at -20°C until use and so the same bacterial concentration and batch was used in each experiment. Graph 7.3 is the average IL-8

stimulated from controls in all experiments and shows that at 10^8 E. coli/ml less IL-8 was stimulated than at 10^6 bacterial/ml from A549 cells. 10^8 E. coli/ml caused the A549 cells in culture to round up and detach from the surface of the cell culture flask which was visible under the inverted microscope. Such an overwhelming concentration of bacteria at 10^8 bacterial/ml probably out competed the A549 cells for the available nutrients causing the cells to detach. At 10^6 bacterial/ml there was an approximate doubling of the IL-8 response which was statistically significant ($p = 0.0002$) and at 10^4 bacterial/ml very little difference was seen compared to the control and this was not significantly different from the control.

Previous studies of lipopolysaccharide (LPS) have shown it is less stimulatory than *U. urealyticum* for IL-8 production from lung fibroblasts (Stancombe *et al.*,1993). The results presented here are from whole live organisms, although LPS is a component of the outer cell wall of these organisms. This may account for their ability to stimulate greater IL-8 concentrations compared to U. urealyticum in these experiments. It is also possible that had we been able to culture greater numbers of U. urealyticum then IL-8 levels would have been comparable to those stimulated by E. coli.

In nearly all assays TNF-α has a significant main effect, raising the IL-8 response. Graphs 7.4 and 7.5 are, respectively, IL-8 values averaged for the higher batch of U. urealyticum and for all E. coli experiments. Although the differences are only marginal this was deliberate. A higher dose of TNF-α would have stimulated IL-8 maximally and prevented any synergisitic effects between assay components from being investigated.

Oxygen also has a significant main effect in all assays. Graphs 7.6 is the IL-8 values for the more concentrated U. urealyticum batch averaged for all controls, and 7.7 is the IL-8 values for all the E. coli assays averaged for all controls. Oxygen concentration generally reduces the IL-8 response, though the degree to which it does this varies between assays. A previous study (Stancombe *et al.*,1993) had shown that lung fibroblasts were unaffected by high oxygen concentration alone. The medium A549 cells grow in maintains its buffering capacity in a 5% CO_2 atmosphere. Without

this CO_2 the medium cannot maintain a steady pH. The cells may be affected by the high oxygen concentration and low pH and be responding with a reduced level of IL-8. Stancombe *et al* 1993 also demonstrated that U. urealyticum in the presence of high oxygen stimulated a greater IL-8 response than U. urealyticum alone. These results are not repeated here.

The presence of TNF-α in a high oxygen concentration increases or has no effect on the IL-8 response compared to when the cells are stimulated by oxygen alone. Graphs 7.8 and 7.9 are, respectively, IL-8 values averaged for the higher batch of U. urealyticum and for all E. coli experiments. Whatever mechanism causes a reduction of IL-8 production in high oxygen it is partially overcome by the presence of TNF-α. There were no synergistic effect of oxygen and TNF-α.

The various clinical treatments these infants may receive in the course of their stay in the unit may have a direct bearing on the lung inflammatory response. A549 cells were cultured with E. coli or U. urealyticum in the presence of antibiotics, surfactants and steroids and the effect on the production of IL-8 investigated. The following discussion of the effects of mediators concentrates on the bacterial concentrations that were stimulatory i.e. 10^5 CFU U. urealyticum/ml and 10^6 E. coli/ml.

Erythromycin is an antibiotic effective against the genital mycoplasmas, but not against E. coli. It is effective in adult chronic airway diseases (Sawakai *et al.*,1986; Kudoh *et al.*,1987) where it has also been shown to play an anti-inflammatory role. It can reduce neutrophil chemotaxis (Nelson *et al.*,1987; Esterley *et al.*,1990), elastolytic activity (Ichikawa *et al.*,1992) and phagocytosis (Hand *et al.*,1990), probably by blocking the mobilisation of calcium ions in the cells. It may also perturb cAMP intracellularly and promote neutrophil apoptosis (Aoshiba *et al* 1997) so neutrophils commit suicide before any damage can be done.

Erythromycin had a significant main effect in the U. urealyticum experiment but did not in the E.coli experiment, which suggests for the U. urealyticum experiment at least, its presence affected the IL-8 response.

The stimulation experiments with erythromycin used a batch of *U. urealyticum* which was too low in concentration to produce an increase in IL-8 production from the A549 cells. The IL-8 values in Graph 7.10 are therefore averaged for all bacterial concentrations as this factor did not influence IL-8 values. The curve does rise over the erythromycin concentrations, but these differences are not significant from the control in a t-test. There are no other significant interactions of erythromycin and any other factor in either experiment.

Given that erythromycin is effective against U. urealyticum it would have been useful to have had a culture of U. urealyticum that had provoked an inflammatory response. This would have allowed for analysis of the effect of erythromycin on the production of IL-8 in the presence of U. urealyticum.

Gentamicin had a significant main effect in both experiments. The raw data is in Tables 10.22 and 10.27. The same batch of *U. urealyticum* was used in these experiments as for the erythromycin experiments so no stimulation of IL-8 response above control levels was seen. Graph 7.11 has the IL-8 values averaged over the different bacterial concentrations as this did not affect IL-8 levels. Gentamicin appears to inhibit the IL-8 compared to controls. However one plate in the triplicate for the controls produced twice the amount of IL-8 as the other two and skews the result. This is reflected in the much higher standard error of the mean in these groups (shown as bold in 10.22, Appendix B). There is a main effect seen with gentamicin ($p = 0.005$), and three other significant interactions, but because of the one skewed result the controls seem very high compared to when gentamicin is present. When this one result is excluded from the analysis there is a no difference between controls and when gentamicin is present.

As gentamicin is not active against U. urealyticum it would have been unlikely to have had any major effect, but we can not rule this out, due to the lack of stimulation of IL-8 at this U. urealyticum concentration.

Gentamicin can be effective against *E. coli* and this particular isolate was known not to be gentamicin resistant. As expected it reduced the IL-8 response in a dose-dependent manner at 10^6 E. coli/ml, shown in Graph 7.12, presumably by preventing *E. coli* growth and lowering the number of bacteria in culture that could stimulate the cells. In the gentamicin experiment the IL-8 levels at 10^8 bacterial/ml were higher than at 10^6 bacterial/ml (results not shown). This would seem to confirm that at 10^8 bacterial/ml there are just too many organisms, but that gentamicin can prevent their multiplication to the extent that the cells get enough of the available nutrients to survive. There was no synergy between gentamicin and TNF-α or oxygen and no other significant interactions.

Surfactant is given intratracheally i.e. directly into the site of the inflammation whereas antibiotics are given systemically. In the surfactant experiments the *U. urealyticum* batch was concentrated enough for stimulation of the A549 cells.

Both surfactants had significant main effects in both experiments. In the U. urealyticum experiments both surfactants had $p < .001$. Graphs 7.13 and 7.14 are respectively Exosurf and Curosurf concentrations for 10^5 CFU U. urealyticum/ml. Both surfactants are inhibitory in a dose dependent manner with Exosurf showing a stronger inhibition than Curosurf at 500ng/ml. When Exosurf is compared to Curosurf in a separate ANOVA the trend of inhibition by Exosurf is stronger than that of Curosurf ($p > 0.001$).

The degree of significance in the E. coli experiments is less than the U. urealyticum experiments and the data is plotted in Graphs 7.15 and 7.16. There is a trend towards inhibition with Exosurf but this does not appear to be true of Curosurf. None of these differences are statistically different from the control which is probably due to the greater variability between plates for this set of assays. It seems unlikely that the difference between the *U. urealyticum* experiments and the *E. coli* experiments reflect that Exosurf works differently in the presence of the different bacteria. If this was the case then the controls without bacteria would both show the same results. Graphs

7.13 to 7.16 all have the IL-8 values in the presence of the varying surfactant concentrations without bacteria. In the *U. urealyticum* experiments there is inhibition even when *U. urealyticum* is not present, but in the *E. coli* experiments there is no IL-8 inhibition. The surfactants are acting differently especially at the highest Exosurf concentration. This may be due to experimenter error, which is always a problem with any assay and if time had permitted this experiment would have been repeated.

The highly significant inhibition by Exosurf in the *U. urealyticum* experiments is not surprising as surfactant lipids have an immunosuppressive role *in vivo* (Shimizu *et al.*,1988) directed against the alveolar macrophage. The two hydrophilic proteins SP-A and SP-D are immunomodulatory facilitating macrophage phagocytosis by opsonisation of inhaled particles (Malhotra *et al.*,1993) and bacteria (Lim *et al.*,1994; Kalina *et al.*,1995). They stimulate proinflammatory cytokines (Eizioni.,1994) and oxygen radicals from alveolar macrophages (van Iwaarden *et al.*,1992) and have been suggested to scavenge LPS which may be important in the prevention of septic shock (Creuwels *et al.*,1997).

The two surfactants produce different inhibitory responses in the U. urealyticum experiments. Both are dose-dependent but Curosurf, which is a natural porcine derived surfactant, barely reduces the IL-8 produced even at high DPPC concentrations. Exosurf, the synthetic surfactant on the other hand reduces the IL-8 response by more than half at the equivalent concentration of DPPC. The two surfactants have quite different components. Curosurf has the natural proteins that are associated with surfactant *in vitro*, whereas Exosurf has only synthetic components which mimic the surface spreading activities of the proteins.

As the lipids are inhibitory and the proteins immunomodulatory it is not surprising that the surfactant with no proteins should produce a significantly larger inhibition of the IL-8 response. As the importance of the epithelial cell response in recruitment of the neutrophils into the lung has now been established the effects of surfactant on the epithelial cells would be worth further investigation.

Dexamethasone is an anti-inflammatory which binds to a specific TATA sequence on DNA and prevents transcription of IL-8 mRNA (Kwon *et al.*,1994). In both the *U. urealyticum* and *E. coli* experiments it had a significant main effect at $p < 0.001$. Graph 7.17 shows that as expected it abolished the stimulated IL-8 response in a dose dependent manner. It also reduced the constitutive response from A549 cells.

7.9.1 Further Study

Further study is definitely required to establish further the effect of these important clinical treatments on other aspects of the epithelial cells role in the inflammatory response. Epithelial cells whilst abundant in the lung and important in the progression of chronic lung disease are not the only cell type involved. Coincubation with monocytes, macrophages and neutrophils would give a fuller picture of the effect of the clinical treatments. A549 cells have been used in experiments to investigate neutrophil migration across the epithelial membrane (Smart *et al.*,1994; Bittleman *et al.*,1995). These cells express ICAM-1 on their surface (Arnold *et al.*,1996), an important molecule which mediates the migration of neutrophils via their LFA-1 integrin. Neutrophil recruitment is an early event in RDS, and important in the progression to chronic lung disease. The effect of these clinical treatments on this event would be very important especially as erythromycin is thought to prevent neutrophil migration into the lungs in adult airways disease. Ideally further study would also involve neonatal cells, and premature ones where possible. Their responses to infection and clinical treatments may possibly be different to adult cells responses.

8. Discussion

Chronic lung disease of prematurity is a major cause of mortality in infants born ≤ 30 weeks gestation. The immaturity of the lung, structurally and biochemically, accounts for many of the problems faced by these infants. The highly compliant chest wall is inadequate to generate sufficient pressure for inflation. The lung has less terminal airspaces, a thick respiratory membrane and higher lung fluid levels, and there is little or no surfactant or anti-oxidant enzymes until approximately 32 weeks gestation. This causes a high surface tension within the lung and the build up of toxic oxygen radicals, both of which result in damage.

Birth at ≤ 30 weeks gestation usually requires ventilation and high oxygen supplements for survival. These contribute to increased barotrauma and toxic oxygen radicals which result in direct tissue damage and the onset of an inflammatory reaction.

The inflammatory response has increasingly been recognised as vital in the progression of chronic lung disease. These infants do not present with an inflammatory response at birth, although there has been recent speculation about in utero inflammation (Ghezzi *et al* 1997), but it is evident within hours by the presence of activated neutrophils and monocytes and increased levels of cytokines. Most studies use bronchoalveolar lavage to measure cells and cytokines but this has proven difficult to standardise. The technique itself involves squirting saline down the endotracheal tube into the lung and aspirating the fluid by suction. There is no one standard protocol between neonatal units and there is no current way of estimating the dilution of the epithelial lung fluid by the saline. None of the proteins so far used - urea, albumin, secretory component of IgA - have been shown to be accurate. They are affected by the inflammatory response and the damage to the epithelial lining caused by barotrauma and enzymic degradation. Clearly a marker of dilution would be advantageous, but so far no one protein has proven reliable.

The lack of a control group also poses problems of interpretation. Infants that are intubated at birth but which subsequently do well are weaned from the ventilator as

soon as possible. We have no indication of the cytokines and cells involved in the resolving inflammatory response.
Comparisons between studies have therefore been complicated but almost all studies have shown high levels of locally produced cytokines and cellular infiltrates.

With the understanding of the role of inflammation in the development of chronic lung disease, studies have been directed at defining the cytokines involved and the role of the infiltrating cells. Early studies of inflammatory mediators in at risk infants showed elevated levels of fibronectin (Watts *et al.*,1992b), TNF-α (Murch *et al.*,1992) and IL-6 (Ng.,1993) associated with the development of chronic lung disease. The role of neutrophils was established by Merrit *et al* (1981), who classified the polymorphs and monocytic cells and recorded that they might have been characteristic of different stages of the disease. Subsequent studies of cellular infiltrates showed that neutrophils were the predominant cell types (Ogden *et al.*, 1984; Arnon *et al* 1993) and it was suggested that neutrophils were the only cells involved in the pathogenesis of chronic lung disease (Arnon *et al* 1993). Macrophages were not seen early in the inflammatory but in resolving cases of RDS and were linked to an eventual "good" outcome.

IL-8 belongs to the α-chemokine family which are specific chemoattractants for neutrophils. It was therefore unsurprising that it was discovered in bronchoalveolar lavage samples of patients with adult respiratory distress syndrome (ARDS) (Donnelly *et al* 1993) a disease characterised by an infiltration of neutrophils into the lung. What was interesting was that it was evident in patients 48 hours before they developed ARDS and not in a control group. This suggested that cytokines may predict the development of disease in at risk groups.

Given that the classical view of neonatal chronic lung disease is an infiltration of neutrophils into the alveolar spaces we conducted a pilot study to establish if IL-8 was detectable in at risk infants (≤ 30 weeks) and if it was of predictive value for those infants that later developed chronic lung disease. 26 infants were enrolled and IL-8

was measured using a radioimmunoassay on admission to the unit and for 72 hours after birth. There was a significantly higher IL-8 level at 24 hours in the group that later went on to develop chronic lung disease. This early rise in IL-8 confirmed that the inflammatory response was provoked almost immediately and that we may have been able to predict the infants that were to progress to chronic lung disease. It also gave rise to the hope that early intervention with anti-inflammatories may prevent the further long term chronic inflammation. Current treatment for chronic lung disease is steroids which are anti-inflammatory but also have serious side-effects. The ability to identify a sub-group 24 hours after birth of the at risk infants who would likely develop chronic lung disease would allow the administration of anti-inflammatories to the group most likely to benefit, without exposing unnecessarily the group that were unlikely to develop chronic lung disease. This would a major benefit in the care of severely preterm infants.

Studies in the mid-90s continued to show an array of inflammatory cytokines that were present in the lungs of ventilated neonates immediately after birth. IL-1β (Jones *et al.*,1996; Liau *et al.*,1996), IL-6 (Bagchi *et al.*,1994), IL-8 (Jones *et al.*,1996) and MIP-1α (Murch *et al.*,1996b) were all found to be higher in infants with RDS compared to those without. The persitence of soluble ICAM-1 (Kotecha *et al.*,1995; Silver *et al.*,1996), TNF-α (Murch *et al.*,1992; Bagchi *et al.*,1994), IL-8 (Groneck *et al.*,1994a; Kotecha *et al.*,1995a), C5a (Groneck *et al.*,1994a), IL-1β (Murch *et al.*,1996b) and MIP-1α (Murch *et al.*,1996b) was correlated to the development of chronic lung disease. However none of these were of predictive value early on. The role of anti-inflammatory cytokines was also investigated with IL-10 shown to be undetectable within the first 96 hours of life in preterm infants, but present in term infants (Jones *et al.*,1996). Neonatal phagocytes have been shown *in vitro* to be functionally deficient (Hill.,1987;) and after birth require time to mature and acquire normal functions (Bellanti., 1979; Usmani et al.,1991; Carre et al.,1992). The persistence of inflammatory mediators may be due in part to a defect in the regulation

of the inflammatory responses by preterm neonates who are unable to down-regulate the inflammatory response leading to tissue damage and chronic lung disease.

In ARDS a recent study has suggested an imbalance between the matrix metalloproteinases (MMPs) and the tissue inhibitors of metalloproteinases (TIMPs) (Ricou., *et al* 1996). These are important in the maintenance of lung architecture and in the repair of damaged tissue. Their imbalance may lead to increased turnover of ECM during disease and play a role in disordered tissue remodelling. This is an area for future research and may lead to new discoveries in the regulation and development of neonatal chronic lung disease.

The role of the macrophage in chronic lung disease has been disputed. Macrophages have an essential role in fibrosis (Kovacs., 1991) and are therefore likely to be involved early on in the inflammatory response. This was shown by Murch *et al* 1996b when they used cell surface markers to identify the macrophage populations present in bronchoalveolar lavage samples. Most studies to this point had used differential stains to discriminate between cell types, but this makes identification of the heterogeneous monocyte/macrophage population difficult. This was confirmed by investigating the bronchoalveolar lavage samples obtained in the clinical studies and performing both differential stain and immunohistochemistry. The differential stain significantly under-estimated the number of macrophages present.

Macrophages and their products promote the development of fibrosis by stimulating fibroblast proliferation and the accumulation of connective tissue. Their early presence suggests that this process is being initiated rapidly and that early intervention in at risk infants may prevent the development of chronic lung disease.

The role of the genital mycoplasmas in the initiation and sustained provocation of the inflammatory response within the lungs has yet to be clearly defined. The genital mycoplasmas *M. hominis* and *U. urealyticum* are common vaginal organisms of pregnant women and are transmitted vertically during birth (Sanchez.,1993). These organisms are capable of provoking an inflammatory response *in vitro* (Stuart.,1993;

Quentmeier *et al.*,1990; Sugama *et al.*,1990; Stancombe *et al.*,1993) and *in vivo* (Groneck *et al.*,1994b; Groneck *et al.*,1996). During an infants treatment in the neonatal unit they are not cultured for routinely and are not susceptible to the commonly administered antibiotics penicillin or gentamicin. If an infant is colonised these organisms are not being eradicated and they may be contributing to the early inflammatory response as well as to the ongoing inflammatory response in infants that later develop chronic lung disease.

Several recent studies have found a strong association between colonisation with the genital mycoplasmas in the lung of preterms and the development of chronic lung disease (Iles *et al.*,1996b; Wang *et al.*,1993b). A causal relationship however, has not been established. Groneck *et al* (1994b & 1996) showed that colonised infants had higher levels of IL-8, IL-1β and IL-6 though they did not correlate this with outcome.

Erythromycin is effective in the treatment of the genital mycoplasmas and the drug of choice in neonates. It is effective in adult chronic airways disease where a neutrophil influx is also important in disease progression (Sawakai *et al.*,1986; Kudoh *et al.*,1987). However it is thought to be acting as an anti-inflammatory in these patients by reducing neutrophil activity and recruitment into the lungs (Ichikawa *et al.*,1992; Nelson *et al.*,1987; Esterley *et al.*,1990).

A randomised, clinical trial of erythromycin was carried out which would allow us to assess;

i) the level of infection in at risk babies
ii) the effect of the genital mycoplasmas on the early inflammatory response
iii) the effect of an antibiotic effective against the genital mycoplasmas on the early inflammatory response
iv) the effect of erythromycin on the early inflammatory response
v) how all of the above factors related to an outcome of chronic lung disease.

Although the trial ran for nearly two years we were unable to recruit our target number of infants. This was most likely due to changes in prenatal management of

mothers threatening preterm labour compared to our two pilot studies. Any mother admitted with the risk of premature labour was given steroids to promote the maturation of the foetal lungs in utero. Corticosteroids stimulate surfactant production, improve lung compliance and decrease the risk or respiratory distress syndrome. Infants are therefore less likely to require ventilation and this may have reduced the number of infants available for our study. Only 9 babies (13%) were infected compared to 30% in the pilot study and as the study used PCR to determine U. urealyticum infection this was likely to be a true reflection of the infection rate. This made analysis of the data in the infected infants difficult. There was no difference in the early inflammatory response between infected and uninfected infants. There was also no difference between infants treated with erythromycin and the control group. Erythromycin is known to be phagocytosed by neutrophils and is thought to promote apoptosis (Aoshiba *et al* 1997), the controlled suicide of cells. In this way it may prevent neutrophils from causing tissue damage. *In vitro* studies have shown that neutrophils from premature infants are functionally immature (Hill.,1987; Eisenfeld *et al.*,1994; Santos *et al.*,1993), with a greatly reduced ability to phagocytose (Falconer *et al.*,1995b). This may prevent them from internalising erythromycin and explain its apparent lack of any anti-inflammatory effect.

In this study infection with the genital mycoplasmas did not correlate with the development of chronic lung disease, nor did it influence the severity of that lung disease as measured by arterial-oxygen differences. Treatment with erythromycin also had no effect on an outcome of chronic lung disease. These organisms may therefore be of less importance than other factors such as ventilation and high oxygen supplements in the development of chronic lung disease.

None of the cytokines measured correlated to outcome. This was in contrast to our pilot study which had clearly shown IL-8 as a predictor of the development of chronic lung disease. Pilot studies are not as strictly controlled as a randomised clinical trial. Although the infants studied during the pilot were sequentially admitted to the unit, there was no attempt made before hand to define the number of infants that would be

required to provide statistically relevant data. The decision was made after 26 infants to analyse the data and significant results were obtained. The goal in the clinical trial was to enrol 90 babies as this would have given us a statistically large enough sample from which to draw conclusions. That we failed to recruit enough numbers may have had some bearing on the results. A pilot study is a necessity to any large trial and it may have been that the infants recruited during the period of the pilot were unrepresentative of the population as a whole.

Another difference between the studies was the definition of chronic lung disease. The pilot study was using a definition of supplemental oxygen at 28 days postnatal age with abnormalities on x-rays. In the erythromycin trial a baby who had an oxygen requirement at 36 weeks corrected gestational age with abnormalities on x-ray was defined in the chronic lung disease group. Only the second definition predicts abnormal findings at follow-up in infants ≤ 30 weeks gestation (Shennan *et al.*,1988).

The cytokines measured < 4 hours after birth did correlate strongly with chorioamnionitis. Chorioamnionitis has been associated with preterm labour, an increased infection in the baby (Hillier *et al.*, 1988) and an outcome of chronic lung disease (Watterberg *et al.*,1996). However, in this study chorioamnionitis was not correlated to lung infection and infants with chorioamnionitis were less likely to be treated with surfactant and had a significantly lower incidence of chronic lung disease. The placentas of these infants were not cultured for genital mycoplasmas so we can not be certain that these infants were already infected in utero. The cytokines in all infants were similar by 24 hours suggesting that chorioamnionitis may provoke an early rise but that in the longer term this has no significance on outcome. The persistence of inflammatory cytokines may be more important in the development of chronic lung disease.

Studies on human populations are always compounded by the individual differences between subjects. It is very rare to get two infants with the same clinical course and the same outcome. For this reason animal models and *in vitro* studies are often very

useful in elucidating disease mechanisms. Natural animal models of chronic lung disease do not occur as premature labour is rare in most species except man, however rabbits, sheep, rats, baboons and guinea pigs can all be electively delivered prematurely. Rabbits, rats and sheep cannot be maintained successfully for prolonged periods but provide models of respiratory distress syndrome. Newborn guinea pigs have relatively mature lungs compared to humans with events such as differentiation and alveolarisation which occur pre-dominantly postnatally in humans occurring prenatally in guinea pigs (Frank.,1983). Non-human primates provide the best model as they can be maintained for longer periods but generally recover without developing chronic lung disease. Within the budget of this study a primate model was not a viable option and instead a cell based model was developed.

A cell model of the inflammatory response was developed as a means to further understand the interactions of lung epithelial cells and mediators used clinically in the treatment of preterm infants. Treatment of preterm infants varies on a case by case basis depending on the clinical needs of the patient.

Infants with RDS will invariably receive surfactant, either natural or synthetic. At the time of this study a clinical trial was underway in the neonatal unit evaluating porcine natural surfactant (Curosurf) and synthetic surfactant (Exosurf). Surfactant is known to decrease the lung surface tension, and contribute to the alveolar stability associated with normal respiration preventing the collapse of the alveoli. Surfactant lipids suppress the immunological activities of alveolar macrophages (AM) (Shimizu *et al.*,1988) whilst the proteins stabilise the alveolar film (Baatz *et al.*,1990; Perez-Gil *et al.*,1992). SP-A has been shown to stimulate proinflammatory cytokine production (Eizioni.,1994) and SP-D can induce production of oxygen radicals from alveolar macrophages (van Iwaarden *et al.*,1992).

Curosurf is derived from the minced lungs of pigs and contains both the lipid and protein parts of surfactant. Exosurf is synthetic and contains lipids but no proteins. Instead it contains two synthesised components hexadecanol and tyloxapol which

mimic the proteins SP-B and SP-C, and which are essential if the surfactant is to form a surface film at the air/water interface.

Exosurf inhibits alveolar macrophage production of IL-1β, TNF-α and IL-6 (Thomassen *et al.*,1994) but not IL-8 (Thomassen *et al.*,1992) and may function as an antioxidant by scavenging oxygen radicals (Ghio *et al.*,1994). Curosurf in contrast inhibits monocyte TNF-α and superoxide anion production (Ciccimarra.,1994), and phagocytosis of *S. aureus* (Speer *et al.*,1991). Exogenous surfactant has been shown to promote endogenous surfactant production (Gerdes *et al.*,1992).

The make-up of these two surfactants suggests they may have different immunomodulatory roles.

Dexamethasone is a steroidal anti-inflammatory given to infants in whom little clinical progress is seen. It is not routinely used in our unit due to its side-effects (Ng.,1993), though it could be argued that if given early in the course of a ventilated infant it reduces inflammatory markers and may prevent the development of chronic lung disease (Groneck *et al.*,1993c; Groneck *et al.*,1993b; Murch *et al.*,1996b). Early and prolonged treatment would seem to be more beneficial in reducing the long term complications (Rastogi *et al.*,1996; Kari *et al.*,1993; Sanders *et al.*,1994).

Another common treatment used is antibiotics. Routinely infants receive gentamicin which is effective against a range of common Gram-negative organisms, but has no known immunomodulatory effects. To assess erythromycin as an anti-inflammatory it too was included in our model of inflammation.

Lung epithelial cells are important in modulating the migration of neutrophils into the lungs. They express cell surface adhesion molecules and can produce IL-8 on stimulation with TNF-α and IL-1β. A human lung epithelial cell line (A549) was cultured *in vitro* and exposed to *U. Urealyticum* and *E. coli* which stimulated IL-8 production. *E. coli* produced a significantly larger response than *U. urealyticum.* Dexamethasone completely blocked the production of IL-8, erythromycin and gentamicin had no direct effect and the surfactants Exosurf and Curosurf also

inhibited the response in a dose dependent manner. Exosurf was much more inhibitory than Curosurf on the epithelial cells. In contrast, in a randomised clinical study of Curosurf vs. Exosurf the infants that received Exosurf had a larger rise in bronchoalveolar lavage IL-8 after 24 hours which was statistically significant. The two groups in the study had no other significant differences in clinical characteristics, although the numbers were small (n = 23). Whilst the cell model reflects changes in the epithelial cells, *in vivo* there are also macrophages and large numbers of neutrophils contributing to the IL-8 present. Exosurf may inhibit epithelial cell production of IL-8 but it may also promote its local production by other cells. Alternatively, Curosurf may be much better at inhibiting IL-8 production from inflammatory cells within the lung and this may relate to its composition which includes proteins.

The further development of this model could include these cell types in co-culture experiments with macrophages and neutrophils. This would reflect more accurately the situation *in vivo*. It would also be of interest to culture cells from preterm neonates although numbers would inevitably be small and a great deal of effort would be required to collect enough samples for analysis. Further assays could investigate the regulation of neutrophil migration across the epithelial membrane (Smart *et al.*,1994; Bittleman *et al.*,1995). A549 cells express ICAM-1 on their surface (Arnold *et al.*,1996), the molecule which mediates the migration of neutrophils. The effect of these clinical treatments on this event would be very important especially as erythromycin is thought to prevent neutrophil migration into the lungs in adult airways disease. Ideally further study would also involve neonatal cells, and premature ones where possible. Their responses to infection and clinical treatments may possibly be different to adult cells responses.

Conclusions

Whilst inflammation is undoubtedly the underlying cause of tissue damage in these infants the early pro-inflammatory cytokines do not appear to provide a marker for

the longer term outcome of chronic lung disease. Colonisation with the genital mycoplasmas does not appear to influence the development of chronic lung disease nor of the early pro-inflammatory response. Recent reports have suggested that neonatal cells are incapable of producing IL-10 (Jones *et al.*,1996), a cytokine which down-regulates the pro-inflammatory cytokines. There is no evidence from this study that infants that develop chronic lung disease do so because of a different early pro-inflammatory response. It may be that neonates who develop chronic lung disease do so because they are unable to down-regulate their inflammatory responses leaving their lungs under persistent immune cell attack. Infants that do not develop chronic lung disease are extubated and we therefore have no knowledge of pro- or anti-inflammatory cytokines that may be present in their lungs. Current investigations in this unit are concentrating on the cytokine IL-10 to measure differences between infants ability to regulate their inflammatory response and its contribution to the development of chronic lung disease.

9. Appendix A: Solutions

Iodination Buffer
0.5M sodium dihydrogen phosphate
pH to 7.4 with sodium hydroxide

Non-stick Buffer
6.05g Trizma Base (Sigma)
2.5g bovine serum albumin
10mls 10% Tween-80
0.5g sodium azide
100mls distilled water
pH to 7.6 and make up to 500mls in distilled water

IL-8 Assay Phosphate Buffer
0.1M PO_4
0.3% BSA
0.05% NaN_3
pH 7.4
0.1% Triton x-100

Precipitating Antibody Solution
IL-8 assay phosphate buffer and 8% PEG were mixed in a 1:1 ratio. To this donkey anti-rabbit was added at 1:20 and normal rabbit serum at 1:200.

Growth Medium (GM) for A549
DMEM (Life Technologies, Cat. No. 22320022)
100 units/ml penicillin/streptomycin (Life Technologies, Cat. No. 15070030)
2mM L-glutamine (Life Technologies, Cat. No. 15032014)
10% fetal bovine serum (Life Technologies, Cat. No.10106, Batch No. 40G3251F)

Freezing Medium (FM) for A549
Add 1% DMSO (Sigma, Cat. No. D2650) to growth medium.

Acid Hemallum Stain
5g haematoxylin in 700 mls of distilled water
50g ammonium alum
0.25g sodium iodate
Dissolve the above with heat then add
300g glycerine
20 mls glacial acetic acid

Normal Human Serum
Whole blood was collected and allowed to clot. The mixture was then spun at 1000g for 10 minutes and the supernatant collected into aliquots and frozen at - 20°C until use.

Fast Red Substrate
Dissolve 8mg of napthol-AS-MX-phosphate (Sigma, Cat. No. N4875) in 0.2 ml N,N, dimethyl formamide (Sigma, Cat. No. D8654)
Add 10 mls 0.1M tris
Adjust pH to 8.2
Add 2.5 mg levamisol (Sigma, Cat. No. L9756)
Immediately prior to staining add 10 mg fast red TR (Sigma, Cat. No. F2768)

Tris Buffered Saline (TBS)
80g NaCl
6.05g Tris
44ml 1N Hcl
1 litre distilled water
Adjust to pH 7.6 and make up to 10 litres in distilled water

10. Appendix B: Raw Data Tables

Chapter 3: IL-8 Assay Development Results

Table 10-1: Results for the Equilibrated Essay.

Values are counts per minute. Stdev = standard deviation; N/A = no sample available; % cv = mean ÷ stdev x 100; % B0 = Counts expressed as a percentage of B0.

	Tc	NSB	B0	19.5	39.1	78.1	156.3	312.5	625	1250	2500
	18353	351	4744	4613	4392	4202	3861	3069	2602	1836	1393
	18027	398	4703	4524	4371	4075	3882	3259	2571	1792	1357
	18068	399	4722	4455	4522	4114	3917	3140	2638	1915	1415
	18133	416	4744	4378	4443	4205	3526	3283	2606	N/A	1366
	18147	418	4670	4605	4482	4373	3853	3171	2546	1931	1356
	18087	409	4523	4418	4286	4281	3653	3275	2593	1919	1305
	18123	436	4551	4539	4329	4056	3727	3152	2411	1864	1314
	17746	386	4538	4441	4359	3993	3618	3180	2479	1806	1579
	17186	393	4748	4528	4493	3981	3733	3183	N/A	1808	1342
	17970	352	4502	4683	4342	3987	3705	3181	2425	1832	1424
average	17984	396	4644	4518	4401	4126	3747	3189	2541	1855	1385
stdev	319.3	27.4	103.1	96.5	78.9	134.5	128.3	66.5	83.1	53.6	78.5
%cv	1.8	6.9	2.2	2.14	1.8	3.3	3.4	2.1	3.3	2.9	5.7
% Tc			25.8								
%B0				**97.8**	**94.8**	**88.9**	**80.7**	**68.7**	**54.7**	**40.0**	**29.8**

Table 10-2: Results for the Dis-equilibrated Assay.

Values are counts per minute. Stdev = standard deviation; N/A = no sample available; % cv = average ÷ stdev x 100; % B0= Counts expressed as a percentage of B0.

	Tc	NSB	B0	19.5	39.1	78.1	156.3	312.5	625	1250	2500
	17814	421.6	5364	4728	4241	3929	3138	2265	1667	1342	1213
	17910	621.9	4993	4572	4337	3828	3170	2389	1720	1360	1021
	18253	420.7	5000	4477	4215	3817	3135	2263	1844	1311	1045
	17690	379.2	4681	4484	4351	3848	3182	2303	1808	1413	1102
	17546	431.4	5142	4462	4227	3912	3056	2310	1798	1277	1135
	17950	470.8	4776	4506	4247	3941	3065	2280	1841	1327	1066
	17852	446.9	4892	4532	4243	3637	3074	N/A	1658	1547	1077
	17691	420.1	4866	4585	4227	3677	3047	2317	1721	1363	1084
	18026	428.9	4956	4584	4249	3747	3224	2302	1776	1310	1110
	179427	436.9	4910	4670.	4247.	3691.	3150.	2296	1668.	1295.	1060.
average	17867	447.8	4958	4560	4258	3803	3124	2303	1750	1354	1091
stdev	199.13	65.39	190.	86.67	46.59	109.8	60.78	37.59	72.54	78.23	54.02
%cv	1.11	14.60	3.84	1.90	1.09	2.89	1.95	1.63	4.14	5.77	4.95
%Tc			**27.75**								
%B0				**91.97**	**85.89**	**76.70**	**63.01**	**46.45**	**35.30**	**27.33**	**22.02**

Table 10-3: Measured and Expected Values of Serially Dilute Tracheal Samples.
Values are pg/ml IL-8. DF = dilution factor. The expected values are calculated by dividing the measured value by the dilution factor.

Sample	Measured Neat Value	DF	Measured Value	Expected Value
1	1303	5	242	260.6
2	1018	2	570	509
		2	284	285
		2	133	142
3	1188	5	254	237.6
4	776	5	158	155.2
5	465	5	66	93
6	1051	2	595	525.5
		2	314	297.5
		2	144	157
7	1178	2	617	589
		2	321	308.5
8	595	5	113	119
9	918	2	529	459
		2	280	264.5
		2	130	140
		2	65	65
10	1617	2	914	808.5
		2	477	457
		2	256	238.5
		2	127	128
11	492	5	91	98.4
12	670	5	118	134
		5	40	23.6
13	983	5	175	196.6
14	1415	5	306	283
		5	58	61.2
15	1438	2	771	719
		2	427	385.5
		2	193	213.5
		2	113	96.5
16	297	2	129	148.5
		2	62	64.5

Table 10-4: Ten Replicates of the R & D Standards

R&D Standard	Tc	NSB	B0	19.5	39.1	78.1	156.3	312.5	625	1250	2500
	18054	427	4814	3999	5161	3893	3016	2439	1711	1359	1051
	18108	484	4886	4613	4414	3669	3026	2327	1703	1371	1118
	17735	411	4961	4595	4169	3829	3365	2362	1796	1262	1201
	17862	468	4956	4643	4272	3699	3281	2289	1774	1377	1026
	17776	425	4941	4618	4268	3787	3130	2378	1633	1328	1069
	17938	350	5045	4619	4297	3842	3077	2264	1565	1305	997
	17975	457	4824	4541	4299	3717	3116	2290	1781	1244	1032
	18365	400	4793	4639	4450	3721	2958	2265	1880	1284	1049
	17838	390	4801	4649	4334	3789	3009	2317	1823	N/A	1129
	17890	379	4716	4927	4367	3797	3014	2368	1808	1358	965
average	17954	419	4873	4584	4403	3774	3099	2329	1747	1320	1063
stdev	185.29	41.9	100.6	229.9	277.9	71.06	130.4	56.46	94.76	49.41	69.19
% cv	1.03	10.0	2.06	5.02	6.31	1.88	4.21	2.42	5.42	3.74	6.50
% Tc			**27.15**								
%B0				**94.06**	**90.34**	**77.44**	**63.59**	**47.80**	**35.85**	**27.10**	**21.82**

Table 10-5: Ten Replicates of the NIBSC Standard

NIBSC Standard	Tc	NSB	B0	19.5	39.1	78.1	156.3	312.5	625	1250	2500
	18110	419	5034	4474	4282	3247	N/A	2486	1687	1315	1101
	18127	434	5113	4462	4373	3696	3106	2157	1787	1209	1007
	18473	421	4990	4609	4398	3794	2934	2293	1696	1293	1007
	18063	395	4900	4529	4338	3600	2979	2344	1740	1207	1036
	18184	529	4813	4679	4111	3693	2912	2316	1589	1236	980
	17777	493	4854	4658	4170	3695	3036	2212	1673	1290	1065
	17249	428	4879	N/A	4321	3643	3123	2162	1572	1257	1056
	17727	426	4748	4355	4344	3760	3019	2211	1590	1325	1076
	17958	530	4837	4425	4137	3661	3031	2114	1667	1277	1046
	18095	411	4916	4322	4234	3876	3078	2074	1692	1278	1148
average	17976.	448.6	4908	4501	4270	3666	3024	2236	1669	1268	1052
stdev	330.52	49.59	109.6	127.5	102.2	167.4	72.74	123.7	68.81	40.95	49.33
% cv	1.84	11.05	2.23	2.83	2.39	4.57	2.41	5.53	4.12	3.23	4.69
% Tc			**27.30**								
% B0				**91.71**	**87.01**	**74.70**	**61.61**	**45.57**	**34.01**	**25.85**	**21.44**

Chapter 4: Results

Table 10-6: IL-8 (μg/L) for All Infants Enrolled on the Study

Time (Hrs)			IL-8 (μg/L)
Mean	**n**	**sem (Range)**	**Median (IQR)**
2.4	16	0.6 (0 - 7.9)	3.4 (1.5 - 13.8)
26.0	21	5.8 (14.8 - 39.6)	18.5 (6.4 - 37.3)
47.6	16	2.3 (40.6 - 59.9)	21.0 (11.1 - 47.3)
71.4	14	19.1 (63.3 - 79.9)	12.3 (3.3 - 43.4)

See also Graph 4.1. sem = standard error of the mean; IQR = interquartile range.

Table 10-7: Interleukin-8 μg/L

Infants divided retrospectively for those that developed CLD and those that did not.

	Chronic Lung Disease		No Chronic Lung Disease	
Time (Hrs)	**n**	**Median (IQR)**	**n**	**Median IQR**
2.4	8	4.1 (2.7 - 18)	8	2.3 (0 - 7.9)
26.0	14	25.0 * (10.3 - 58.1)	7	4.7 * (3.3 - 20.7)
47.6	10	21.0 (16.9 - 44.8)	6	28.0 (4.2 - 71.4)
71.4	9	11.8 (3.0 - 31.4)	5	16.2 (2.2 - 61.4)

*See also Graph 4.2. * = $p < 0.01$; Mann-Whitney U*

Table 10-8: Cells in Bronchoalveolar Lavage

Values are x 10^5 medians (range).

Time (Hrs)	Cells/ml x 10^5/ml	Neutrophils x 10^5/ml	Macrophages/ml x 10^5/ml	Epithelial Cells x 10^5/ml
2.5	4.1 (1.0 - 15.4)	0.4 (0 - 0.9)	0.06 (0 - 0.4)	1.9 (0.5 - 6.5)
26.0	7.0 (1.8 - 18.3)	3.8 (0.6 - 9.7)	1.0 (0.1 - 3.7)	0.5 (0.02 - 1.9)
47.6	3.1 (9.0 - 81.0)	2.2 (1.1 - 6.0)	0.4 (0.04 - 0.6)	0.03 (0 - 0.2)
71.4	5.8 (2.2 - 12.3)	2.9 (1.5 - 10.1)	0.8 (0.4 - 1.3)	0.4 (0 - 1.2)

See also Graph 4.3

Table 10-9: Cell Results Chronic Lung Disease vs. No Chronic Lung Disease

Values are x 10^5 median (range).

Sample Time (Hrs)		2.5	26	47.6	71.4
CLD	**Total Cells/ml**	5.9 (1.4,18.2)	7.1 (1.8,18.1)	3.1 (1.6,9.3)	4.4 (1.2,9.8)
	Neutrophils/ml	0.2 (0,0.4)	3.2 (0.7,9.1)	2.2 (0.3,7.2)	2.2 (0.9,7.7)
	Macrophages/ml	0.06 (0,0.3)	1.1 (0.2,3.6)	0.3 (0.01,0.8)	0.5 (0.2,1.7)
	Epithelial Cells/ml	5.0 § (1.1,17.8)	0.4 (0.2,4.8)	0.03 (0,0.4)	0.2 (0,0.7)
No CLD	**Total Cells/ml**	3.0 (0,5.7)	7.0 (1.6,39.0)	3.1 (0.6,9.9)	11.0 (5.5,14.5)
	Neutrophils/ml	0.05 (0,2.2)	5.4 (0.1,19.5)	1.6 (0,5.3)	7.9 (4.2,12.5)
	Macrophages/ml	0.06 (0,0.5)	0.2 (0,12.4)	0.2 (0,0.7)	1.0 (0.6,1.2)
	Epithelial Cells/ml	0.8 § (0,4.0)	0.5 (0,1.5)	0.07 (0,3.6)	1.1 (0.3,2.3)

§ $p < 0.05$ Mann Whitney U test.

See also Graphs 4.4 to 4.7

Table 10-10: Cytokine Data. Medians (interquartile range) for all infants enrolled on the study. See also Graph 5.7.

	Sample Time (Hours Postnatally)					
	Pre-S	**Post-S**	**24 - 48**	**48 - 72**	**72 - 96**	**96 - 120**
IL-8 μg/L	5.8 (1.7 - 25.3)	9.0 (2.6 - 19.6)	28.3 (10.6 - 64.2)	39.9 (19.6 - 90.0)	42.9 (9.7 - 96.8)	50.4 (7.6 - 88.0)
TNF-α ng/L	0 (0 - 0)	0 (0 - 0)	0 (0 - 31)	0 (0 - 68)	0 (0 - 171)	0 (0 - 160)
IL-1β ng/L	3.6 (0 - 122)	56.2 (17 - 173)	213.6 (55 - 639)	297.6 (140 - 833)	258.4 (106 - 834)	407.5 (41 - 973)

Table 10-11: Cell Data. Medians (interquartile range) for all infants enrolled on the study. See also Graph 5.8

	Sample Time (Hours Postnatally)				
Cells/ml x 10^5	**Pre-S**	**Post-S**	**24 - 48**	**48 - 72**	**72 - 96**
Total Cells	1.4 (0.8 - 4.0)	4.5 (2.4 - 5.8)	5.6 (3.1 - 10.4)	5.2 (3.0 - 9.7)	7.0 (3.9 - 10.7)
Neutrophils	0.2 (0 - 0.9)	3.0 (0.2 - 6.2)	3.0 (0.6 - 6.6)	4.2 (0 - 9.0)	3.0 (0.3 - 6.5)
Macrophages	0.008 (0 - 1.3)	0.3 (0 - 7.1)	0.4 (0 - 0.9)	0.3 (0 - 1.6)	0.7 (0.02 - 1.8)
Epithelial Cells	0.6 (0.2 - 2.4)	0.05 (0 - 0.8)	0.002 (0 0.4)	0.05 (0 - 0.5)	0.05 (0 - 0.7)

Table 10-12: Cytokine Data. Medians (interquartile range) for infants divided into the group that received erythromycin (E) and the non-treated group (NT). See Graphs 5.6 and 5.7.

Hours	Pre-S		Post-S		24 - 48		48 - 72		72 - 96	
	E	NT	E	NT	E	NT	E	NT	E	NT
IL-8 µg/L	5.8 (1.4 - 23.6)	5.2 (2.1 - 25.1)	10.1 (4.0 - 19.7)	8.3 (2.2 - 17.4)	11.8 (8.0 - 39.8)	38.8 (14.6 - 77.4)	46.4 (18.0 - 139.9)	33.7 (18.2 - 56.4)	89.8 (12.0 - 146.0)	28.4 (8.3 - 74.0)
TNF-α ng/L	0 (0 - 39.9)	0 (0 - 0)	0 (0 - 10.8)	0 (0 - 0)	0 (0 - 55.8)	0 (0 - 28.8)	0 (0 - 69.4)	0 (0 - 23.6)	0 (0 - 208.3)	0 (0 - 0)
IL-1β ng/L	18.0 (0 - 230)	0 (0 - 82)	41.5 (0 -114)	75.7 (0 - 224)	113.1 (47 - 727)	348.0 (139 - 689)	242.0 (93 - 867)	457.6 (161 - 937)	640.0 (96 - 937)	229.7 (135 - 668)

Table 10-13: Cell Data. Medians (interquartile range) for infants divided into the group that received erythromycin (E) and the non-treated group (NT). See Graphs 5.8 to 5.11.

Hours	Pre-S		Post-S		24 - 48		48 - 72		72 - 96	
Cells/ml x 10^4	E	NT	E	NT	E	NT	E	NT	E	NT
Total Cells	16 (8 - 38.5)	13 (8 - 43)	48 (30 - 84)	39 (15.5 - 62.5)	61 (40 - 114.5)	56.0 (30 - 102)	70 (28 - 143)	48 (30 - 86)	92 (44.5 - 114)	64 (20.8 - 88)
Neutrophils	2.0 (0 - 14.0)	0.7 (0 - 8.1)	25.7 (1.2 - 54.9)	30.8 (2.7 - 51.6)	33.2 (1.5 - 80.5)	23.2 (10 - 66.0)	52.4 (0 - 106)	26.7 (0 - 59.8)	37.6 (8.1 - 65.9)	15.8 (2.8 - 45.5)
Macrophages	0.2 (0 -1.7)	0.02 (0 - 1.0)	2.4 (0.5 - 5.9)	2.7 (0 - 11.4)	3.9 (0 - 8.6)	4.1 (1.4 - 9.5)	7.3 (0 - 24.3)	1.8 (0 - 5.8)	6.5 (0.7 - 26.8)	6.4 (0.2 - 16.1)
Epithelial Cells	4.4 (1.0 - 28.2)	8.7 (3.6 - 23.5)	1.1 (0 - 9.6)	0.5 (0 - 7.2)	0 (0 - 4.5)	0.04 (0 - 4.0)	0.4 (0 - 4.7)	0.4 (0 - 3.6)	0.2 (0 - 3.6)	0.6 (0 - 10.8)

*Table 10-14: Medians (interquartile range). * = $p < 0.05$, ** = $p < 0.01$. See Graphs 5.12 to 5.13.*

Hours	Pre-S		Post-S		24 - 48		48 - 72		72 - 96	
	I	NI	I	NI	I	NI	I	NI	I	NI
IL-8 μg/L	7.7 (4.2 - 6.6)	3.6 (0 - 12.2)	9.0 (1.7 - 15.2)	8.3 (3.3 - 20.1)	12.1 (3.7 - 114.9)	29.7 (9.9 - 71.4)	25.2 * (3.5 - 41.8)	47.5 * (22 - 112.8)	18.2 (6.4 - 47.9)	72.5 (14 - 105.1)
TNF-α ng/L	0 (0 - 44.8)	0 (0 - 0)	0 ** (0 - 71.0)	0 ** (0 - 0)	46.2 (0 - 59.0)	0 (0 - 28.8)	0 (0 - 68.4)	0 (0 - 60.9)	0 (0 - 46.2)	0 (0 - 227.4)
IL-1β ng/L	38.2 (0 - 618.6)	1.8 (0 - 121.2)	43.9 (22 - 69.5)	56.2 (0 - 172.8)	139.1 (78 - 770.5)	200.5 (47 - 796.0)	225.8 (43 - 543.9)	260 (118 - 900.5)	207.7 (0 - 635)	601.6 (141 - 931.3)

*Table 10-15: Medians (interquartile range) * = $p < 0.05$, ** = $p < 0.01$. See Graphs 5.14 to 5.17.*

Hours	Pre-S		Post-S		24 - 48		48 - 72		72 - 96	
Cells/ml x 10^4	I	NI	I	NI	I	NI	I	NI	I	NI
Total Cells	8 * (2 - 24)	17 * (10 - 43)	66 (30 - 80)	48 (19 - 84)	68 (54 - 134)	56 (27 - 79)	39 (29.5 - 124)	60 (29 - 98.5)	58 (37 - 98.5)	73 (38.5 - 114)
Neutrophils	1.9 (0 - 18.7)	0.1 (0 - 5.9)	51 (15.3 - 73)	19.3 (0.05 - 51.8)	43.7 (0 - 89.4)	29.8 (6.1 - 61.3)	33 (6.0 - 94.1)	35.3 (0 - 87.6)	31.5 (0.3 - 54.1)	29.9 (3.1 - 64.8)
Macrophages	0.08 (0 - 1.4)	0 (0 - 0.8)	2.7 (1.0 - 5.4)	1.4 (0 - 7.1)	7.8 (0 - 9.4)	3.4 (0.6 - 9.2)	3.8 (0.4 - 17.3)	4.0 (0 - 18.1)	2.6 (0.09 - 15)	7.8 (0.8 - 35.8)
Epithelial Cells	1.0 ** (0 - 3.6)	11 ** (4 - 37.3)	2.6 (0 - 16.7)	0.09 (0 - 7.8)	4.0 (0 - 6.8)	0 (0 - 0.6)	0.1 (0 - 5.0)	0.6 (0 - 5.4)	2.4 (0.1 - 8.3)	0.4 (0 - 4.8)

Table 10-16: Cytokine Data. Medians (interquartile range) for infants divided into the group that developed CLD (DC) and the group that did not (NC). See Graphs 5.18 to 5.19.

Hours	Pre-S		Post-S		24 - 48		48 - 72		72 - 96	
	DC	NC	DC	NC	DC	NC	DC	NC	DC	NC
IL-8 μg/L	2.4 ** (0 - 4.9)	8.3 ** (3.4 - 49.7)	10.4 (2.6 - 21.3)	13.3 (3.3 - 20)	28.7 (11 - 85.2)	40.1 (10.9 - 73)	54.9 (33 - 147)	40.3 (19.6 - 90)	73.2 (9.1 - 161)	36.5 (10.8 - 76)
TNF-α ng/L	0 (0 - 0)	0 (0 - 0)	0 (0 - 0)	0 (0 - 0)	0 (0 - 24.5)	0 (0 - 39.6)	0 (0 - 30.5)	0 (0 - 68)	0 (0 - 242.7)	0 (0 - 23.1)
IL-1β ng/L	0 ** (0 - 4.8)	17 ** (0 - 222.2)	51.2 (9.8 - 183)	84.6 (26.3 - 208)	226 (40.6 - 784)	374.0 (73.2 - 771)	656 (98.1 - 825)	297.6 (198 - 1001)	425.3 (96.6 - 760)	229.7 (133 - 950.8)

Table 10-17: Cell Data. Medians (interquartile range) for infants divided into the group that developed CLD (DC) and the group that did not (NC). See Graphs 5.20 to 5.23

Hours	Pre-S		Post-S		24 - 48		48 - 72		72 - 96	
Cells/ml x 10^4	DC	NC	DC	NC	DC	NC	DC	NC	DC	NC
Total Cell	19 (9 - 54.5)	14 (8 - 38)	62 (33 - 132)	42 (30 - 68)	58.7 (24 -124)	56 (39 - 83)	72 (32 - 88)	48 (28 - 142)	85 (47.8 - 107)	46 (35 - 97)
Neutrophils	0 (0 - 6.5)	2.4 (0 - 16.8)	34.6 (0 - 61.9)	36.5 (12.1 - 52.9)	32.8 (10.8 - 65.5)	47.1 (16.8 - 75)	55.5 (1.9 - 88)	29.5 (0 - 122)	26.6 (16.5 - 62.9)	34.5 (12.8 - 61.4)
Macrophages	0 (0 - 0.9)	0.2 (0 - 1.6)	3.0 (0 - 12.4)	2.7 (0 - 6.8)	5.1 (1.3 - 8.9)	6.3 (1.9 - 9.4)	3.7 (0 - 9.7)	2.8 (0 - 19)	8.2 (0.2 - 49.1)	3.8 (2.1 - 13.3)
Epithelial Cells	11 (0.4 - 39.8)	4.2 (2.5 - 18)	0 (0 - 3.4)	1.7 (0 - 6.8)	0.2 (0 - 5.0)	0.02 (0 - 3.6)	0.7 (0 - 4.4)	0 (0 - 0.7)	2.9 (0 - 8.1)	0.4 (0 - 7.9)

*Table 10-18: Cytokine Data. Medians (interquartile range) * = $p < 0.05$, ** = $p < 0.01$. See Graphs 5.24 to 5.25*

Hours	Pre-S		Post-S		24 - 48		48 - 72		72 - 96	
	S	NS	S	NS	S	NS	S	NS	S	NS
IL-8 μg/L	3.1 ** (0 - 11.1)	9.5 ** (5.8 - 46.7)	10.4 (3.0 - 20.7)	8.7 (1.7 - 13.8)	31.9 (11 - 71.4)	13.1 (5.8 - 34.8)	40.4 (20 - 91.5)	24.7 (7.9 - 45.2)	47.9 (9.8 - 100.7)	33.3 (7.3 - 96.0)
TNF-α ng/L	0 (0 - 0)	0 (0 -19.1)	0 * (0 - 0)	0 * (0 -47.7)	0 (0 - 27.7)	21.7 (0 -53.8)	0 (0 - 64.4)	0 (0 -69.0)	0 (0 - 141.9)	0 (0 -260.4)
IL-1β ng/L	0 * (0 - 29.2)	61.0 * (0 - 236.8)	62.7 (8.4 - 146.8)	53.7 (24 - 563.4)	188.5 (47- 736.8)	414 (107 - 622.1)	253.6 (107.6 - 800)	617.6 (237 - 1001)	288.7 (108 - 873.4)	194.8 (80 - 906.7)

*Table 10-19: Cell Data. Medians (interquartile range). * = $p < 0.05$, ** = $p < 0.01$. See Graphs 5.30 to 5.33*

Hours	Pre-S		Post-S		24 - 48		48 - 72		72 - 96	
Cells/ml x 10^4	S	NS	S	NS	S	NS	S	NS	S	NS
Total Cells	13 (8 - 33.5)	24 (8 - 50)	48 (30 - 66.5)	38 (13.5 - 86)	56.0 (27.5 - 86)	82 (43.1 - 134)	48 (28 - 100)	72 (44 - 94)	76 (43 - 110)	36 (20 - 108)
Neutrophils	0 ** (0 - 4.7)	5.9 ** (0.9 - 31.1)	27.5 (0.2 - 49.0)	30.8 (10 - 68.1)	29.9 (6.1 - 64.6)	29.8 (0.1 - 97.2)	28 (0 - 94.1)	61.9 (41 - 86.5)	33.2 (6.4 - 66.3)	15.8 (0 - 45.5)
Macrophages	0 ** (0 - 0.7)	1.4 ** (0 - 2.7)	2.3 (0 - 6.9)	3.1 (0 8.4)	3.6 (1.2 - 7.8)	9 (0 - 10.6)	2.7 (0 - 15.5)	3.9 (1.8 - 10.4)	7.3 (1.0 - 25.0)	3.4 (0 - 16.1)
Epithelial Cells	9.7 (1.3 - 26.1)	4 (2.7 - 17.8)	1.8 (0 - 8.2)	0.2 (0 - 6.2)	0 (0 - 2.9)	1.6 (0 - 4.3)	0.04 (0 - 4.4)	0.7 (0.3 - 7.5)	0.6 (0 - 8.0)	0.4 (0 - 2.0)

Chapter 7: Ureaplasma urealyticum Results

Table 10-20: Values are interleukin-8 pg/ml.

> = out with the range of the standard curve. N/T = not tested. stdev = standard deviation. 95 % = 95% confidence interval. % inc = the percentage increase from 0 pg/ml TNF-α. See also Graph 7.1.

	TNF-α Concentration pg/ml															
	2000	**1000**	**500**	**250**	**125**	**62.5**	**31.3**	**15.6**	**7.8**	**3.9**	**2**	**1**	**0.5**	**0.3**	**0.1**	**0**
	>	>	>	>	828	512	358	320	200	189	194	178	201	217	240	227
	>	>	>	>	853	498	415	274	233	211	182	189	222	218	286	251
	>	>	>	>	854	424	385	319	238	226	230	232	212	227	264	219
	>	>	>	>	947	579	464	376	NT	NT	257	235	223	214	292	227
	>	>	>	>	1022	598	438	349	236	241	207	NT	217	219	266	244
	>	>	>	>	843	617	424	344	246	252	233	238	242	243	267	222
	>	>	>	>	841	539	368	246	213	187	191	150	256	169	186	236
	>	>	>	>	844	449	358	250	238	170	194	165	211	187	225	235
	>	>	>	>	820	491	335	278	218	186	227	198	187	184	224	213
mean					872	523	394	306	228	208	213	198	219	209	250	230
st dev					67.1	66	43.3	46.2	15.7	29.5	25.1	33.8	20.6	23.6	34.1	12.2
95%					43.8	43.2	28.3	30.2	10.2	19.3	16.4	22.1	13.4	15.4	22.3	8
% inc					379	227	171	133	99	90	93	86	95	91	109	100

Table 10-21: Mean IL-8 pg/ml results from triplicate plates of A549 cells stimulated with U. urealyticum in the presence of erythromycin.
Sem = standard error of the mean.

Ureaplasma Concentration/ ml	**2.4 x 10^4**				**2.4 x 10^3**				**2.4 x 10^2**				**0**			
Erythromycin concentration µg/ml	**10**	**1**	**0.1**	**0**	**10**	**1**	**0.1**	**0**	**10**	**1**	**0.1**	**0**	**10**	**1**	**0.1**	**0**
Control	788	760	758	718	622	720	728	670	720	763	668	653	757	755	725	627
sem	46	22	53	58	58	62	58	37	59	12	54	66	71	63	91	64
TNF-α	858	862	812	821	830	847	804	759	827	923	805	775	796	797	810	800
sem	124	46	71	78	62	31	35	77	34	50	55	85	13	51	27	57
Oxygen	273	297	293	232	285	275	275	264	297	293	289	257	263	282	269	247
sem	11	28	23	27	19	14	6	31	15	38	12	37	16	25	22	22
Oxygen+TNF-α	345	336	328	307	358	335	321	303	344	313	303	305	412	322	312	318
sem	7	16	3	17	3	29	8	12	28	16	1	29	23	42	28	64

Table 10-22: Mean IL-8 pg/ml results from triplicate plates of A549 cells stimulated with Ureaplasma urealyticum in the presence of gentamicin. Sem = standard error of the mean. Results in bold have very high variation due to one triplicate in the controls producing very high IL-8 levels.

Ureaplasma Concentration/ ml	**2.4 x 10^4**				**2.4 x 10^3**				**2.4 x 10^2**				**0**			
Gentamicin concentration µg/ml	**10**	**1**	**0.1**	**0**	**10**	**1**	**0.1**	**0**	**10**	**1**	**0.1**	**0**	**10**	**1**	**0.1**	**0**
Control	721	663	902	**1057**	664	557	891	**1034**	642	682	789	**955**	720	548	815	684
sem	48	53	184	**302**	68	62	156	**327**	114	30	63	**273**	75	87	108	14
TNF-α	825	712	897	818	852	772	929	760	951	711	912	818	871	726	846	754
sem	140	122	58	77	66	81	87	60	77	69	47	68	114	104	89	6
Oxygen	273	277	309	245	266	294	279	254	297	302	315	297	269	269	310	299
sem	37	11	29	14	14	9	16	13	7	10	6	6	8	9	11	38
Oxygen+TNF-α	326	356	345	353	342	373	361	375	325	383	379	369	318	353	335	345
sem	14	21	3	21	13	6	10	45	13	18	24	20	19	10	17	17

Table 10-23: Mean IL-8 pg/ml results from triplicate plates of A549 cells stimulated with Ureaplasma urealyticum in the presence of Exosurf. Sem = standard error of the mean. Bold values have very high sem.

Ureaplasma Concentration/ ml	**8 x 10^5**				**8 x 10^4**				**8 x 10^3**				**0**			
Exosurf (DPPC) concentration ng/ml	**500**	**50**	**5**	**0**	**500**	**50**	**5**	**0**	**500**	**50**	**5**	**0**	**500**	**50**	**5**	**0**
Control	883	1859	1901	2011	630	1105	1177	1204	509	827	**763**	1198	439	732	859	1173
sem	36	45	357	445	30	116	114	146	44	67	**88**	219	43	78	132	237
TNF-α	765	1377	1759	1649	523	1011	1320	1460	553	880	1165	1477	606	1033	1049	1161
sem	80	62	124	164	54	72	191	373	79	122	82	409	90	164	117	133
Oxygen	583	1593	1585	1668	416	886	937	1171	384	761	757	913	344	718	760	877
sem	19	72	280	234	34	111	99	204	21	93	89	163	28	123	79	142
Oxygen+TNF-α	604	1305	1477	1564	525	1036	1123	1283	433	836	999	1085	437	959	1071	960
sem	28	266	113	163	68	122	54	61	39	68	82	98	35	133	191	70

Table 10-24: Mean IL-8 pg/ml results from triplicate plates of A549 cells stimulated with Ureaplasma urealyticum in the presence of Curosurf. Sem = standard error of the mean.

Ureaplasma Concentration/ ml	**8 x 10^5**				**8 x 10^4**				**8 x 10^3**				**0**			
Curosurf (DPPC) concentration ng/ml	**500**	**50**	**5**	**0**	**500**	**50**	**5**	**0**	**500**	**50**	**5**	**0**	**500**	**50**	**5**	**0**
Control	1350	1482	1546	1621	899	1041	1091	1140	924	875	869	1097	903	939	892	1022
sem	173	34	220	167	19	60	94	10	53	62	66	17	73	136	51	34
TNF-α	1489	1331	1615	1790	1118	1091	1233	1233	973	1023	960	1094	1106	1021	1009	1167
sem	163	135	116	15	73	62	22	131	40	72	71	18	83	61	129	24
Oxygen	1361	1444	1413	1605	847	876	975	919	698	783	876	1035	759	699	751	981
sem	21	68	81	58	78	80	20	109	61	49	55	134	95	32	32	109
Oxygen+TNF-α	1139	1132	1135	1411	859	905	912	962	791	855	862	837	788	987	809	1037
sem	74	52	105	170	60	58	103	107	84	50	49	82	17	77	99	133

Table 10-25: Mean IL-8 pg/ml results from triplicate plates of A549 cells stimulated with Ureaplasma urealyticum in the presence of Dexamethasone. Sem = standard error of the mean.

Ureaplasma Concentration/ ml	**1.4 x 10^5**				**1.4 x 10^4**				**1.4 x 10^3**				**0**			
Dexamethasone concentration µg/ml	**10**	**1**	**0.1**	**0**	**10**	**1**	**0.1**	**0**	**10**	**1**	**0.1**	**0**	**10**	**1**	**0.1**	**0**
Control	359	332	506	1267	285	222	319	815	227	225	323	713	224	235	342	827
sem	15	48	33	258	15	30	21	176	15	10	20	23	19	20	51	84
TNF-α	343	327	503	1442	297	269	388	1107	273	281	357	919	257	264	384	859
sem	18	16	2	88	25	22	20	110	15	5	37	109	33	10	10	89
Oxygen	211	219	258	407	177	180	209	309	192	183	181	285	204	194	182	280
sem	20	27	10	23	10	34	17	16	6	24	21	33	17	12	16	20
Oxygen+TNF-α	251	269	269	465	217	220	225	428	167	194	199	374	188	183	211	365
sem	23	7	17	30	11	13	26	1	24	14	29	6	24	4	23	22

Table 10-26: Mean IL-8 pg/ml results from triplicate plates of A549 cells stimulated with E. coli in the presence of Erythromycin. Sem = standard error of the mean.

***E. coli* Concentration/ml**	**1 x 10^6**				**1 x 10^4**				**0**			
Erythromycin concentration µg/ml	**10**	**1**	**0.1**	**0**	**10**	**1**	**0.1**	**0**	**10**	**1**	**0.1**	**0**
Control	1539	1435	1504	1499	757	756	905	804	748	787	785	797
sem	216	75	99	257	56	74	75	46	40	38	73	46
TNF-α	1951	1588	1627	1777	885	899	815	876	851	933	996	841
sem	99	38	27	262	53	41	59	46	98	42	57	25
Oxygen	1438	1281	1284	1520	800	565	608	685	700	696	640	689
sem	177	34	144	112	70	57	46	25	6	117	22	41
Oxygen+TNF-α	1623	1540	1576	1711	848	760	765	840	877	699	708	721
sem	88	154	157	141	61	36	51	83	47	54	39	27

Table 10-27: Mean IL-8 pg/ml results from triplicate plates of A549 cells stimulated with E. coli in the presence of gentamicin. Sem = standard error of the mean.

***E. coli* Concentration/ml**	**1 x 10^6**				**1 x 10^4**				**0**			
Gentamicin concentration µg/ml	**10**	**1**	**0.1**	**0**	**10**	**1**	**0.1**	**0**	**10**	**1**	**0.1**	**0**
Control	1586	1487	1688	1864	820	744	781	861	763	747	787	857
sem	108	108	215	189	67	24	29	72	49	33	46	86
TNF-α	1804	1463	2232	2116	836	928	980	1021	961	1091	1067	1051
sem	174	79	276	136	70	30	107	103	63	8	55	96
Oxygen	1009	925	1043	1199	465	565	531	576	475	549	564	607
sem	58	25	67	47	61	55	45	20	31	81	10	43
Oxygen+TNF-α	1326	1448	1353	1492	729	723	760	744	668	707	801	751
sem	70	75	179	12	108	53	68	13	40	44	13	7

Table 10-28: Mean IL-8 pg/ml results from triplicate plates of A549 cells stimulated with E. coli in the presence of Curosurf. Sem = standard error of the mean.

***E. coli* Concentration/ml**	**1 x 10^6**				**1 x 10^4**				**0**			
Curosurf concentration ng/ml	**500**	**50**	**5**	**0**	**500**	**50**	**5**	**0**	**500**	**50**	**5**	**0**
Control	2437	2361	2752	2240	960	915	939	859	765	668	655	755
sem	52	147	271	171	48	132	39	24	69	111	29	86
TNF-α	2637	2745	2709	2481	1288	985	1133	1024	991	835	875	917
sem	52	309	133	131	162	29	61	57	60	60	68	69
Oxygen	2401	2480	2291	2316	737	579	671	732	523	425	496	529
sem	231	169	427	88	12	43	24	154	21	13	40	57
Oxygen+TNF-α	2536	2752	2151	2717	823	799	808	876	761	795	767	807
sem	91	245	208	171	34	28	147	56	19	66	60	44

Table 10-29: Mean IL-8 pg/ml results from triplicate plates of A549 cells stimulated with E. coli in the presence of Exosurf. Sem = standard error of the mean.

***E. coli* Concentration/ml**	**1×10^6**				**1×10^4**				**0**			
Exosurf concentration ng/ml	**500**	**50**	**5**	**0**	**500**	**50**	**5**	**0**	**500**	**50**	**5**	**0**
Control	2352	2023	2341	2452	1013	741	1107	1048	1056	675	625	725
sem	400	351	304	46	100	30	141	52	145	55	20	47
TNF-α	2701	2623	2723	2901	1113	1207	1357	1211	1287	1032	1180	1232
sem	244	39	525	377	75	120	85	41	37	86	83	31
Oxygen	2528	2621	2595	2141	875	812	731	876	768	556	571	565
sem	225	229	104	160	55	66	20	115	52	28	46	43
Oxygen+TNF-α	2602	2940	2567	2432	1024	837	871	915	1137	940	948	973
sem	288	168	112	132	110	40	52	36	47	38	51	94

Table 10-30: Mean IL-8 pg/ml results from triplicate plates of A549 cells stimulated with E. coli in the presence of dexamethasone. Sem = standard error of the mean.

***E. coli* Concentration/ml**	**1×10^6**				**1×10^4**				**0**			
Dexamethasone concentration μg/ml	**10**	**1**	**0.1**	**0**	**10**	**1**	**0.1**	**0**	**10**	**1**	**0.1**	**0**
Control	275	163	216	562	144	121	171	311	137	161	192	332
sem	23	13	10	24	15	11	32	31	20	26	27	44
TNF-α	334	190	288	676	159	165	211	416	148	205	222	406
sem	4	26	15	39	18	17	15	50	10	31	35	51
Oxygen	518	272	401	1104	233	239	278	593	236	237	277	559
sem	28	27	31	30	34	34	30	7	30	24	33	11
Oxygen+TNF-α	626	342	454	1360	320	260	320	776	316	316	392	770
sem	8	11	17	64	13	22	11	36	18	41	40	46

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12. Publications

The Lancet Vol 343, March 19, 1994 p729

Interleukin-8 in bronchoalveolar lavage samples as a predictor of chronic lung disease in premature Infants.

SIR - Donnelly *et al*[1] reported that the concentration of the neutrophil activating and chemotactic cytokine, interleukin-8 (IL-8), was greater in bronchoalveolar lavage (BAL) samples from trauma patients who went on to develop adult respiratory distress syndrome than from patients who did not. The IL-8 could be detected on admission and before lung damage was clinically evident. It is now recognised that there is a significant pulmonary inflammatory reaction in the development of chronic lung disease (CLD) in premature infants[2]. Many studies have reported the presence of large amounts of neutrophils in BAL samples from premature intubated infants,[3,4] and IL-8 has been shown to be the major chemotactic factor in the lungs of infants at risk of CLD[5]. We wished to test the hypothesis that IL-8 was associated with the development of CLD in premature infants.

BAL samples were obtained daily for 7 days at the time of normal endotracheal suction from 12 consecutive newborns who were born between 23 and 30 weeks' gestation (median 27). The samples were kept at 4°C (mean 1.9 h) until mucus was removed by straining the sample through sterile gauze and the cells were removed by centrifugation at 400g for 10 min. Aliquots of the supernatant were taken and immediately frozen at -70°C. IL-8 was measured by a specific radioimmunoassay (detection limit of 50ng/L, intra-assay variation of <5%, inter-assay variation of <13%).

7 infants (58%) developed CLD (defined as an oxygen requirement at 28 days of age). 2 of these did not receive surfactant as they initially were not diagnosed as having respiratory distress syndrome. 5 infants (42%) did not develop CLD. 3 of these received surfactant because they were initially diagnosed as having respiratory distress syndrome. All infants from both groups survived. IL-8 concentrations the day after admission, taken at a mean age 21.7 hours (range 14.6-29.2 hours). A higher concentration of IL-8 was found in those infants who went on to develop CLD than in those who did not (p=0.042, Mann-Whitney *U* test):

	Median	Range
Infants later developing CLD	41.7ug/L	2.3 - 351.9ug/L
Infants that did not develop CLD	5.7ug/L	0.4 - 20.7ug/L

It seems possible that IL-8 in BAL may be a useful very early prognostic indicator for the development of CLD in premature infants.

We thank Dr R W Kelly, MRC Reproductive Biology Unit, Centre for Reproductive Biology, Edinburgh for the gift of the IL-8 assay reagents.

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1. Donnelly SC, Strieter RM, Kunkel SL, Walz A, Robertson CR, Carter DC *et al.* Interleukin-8 and development of adult respiratory distress syndrome in at risk patient groups. Lancet 1992; 341:643-7.
2. Robertson B . The evolution of neonatal respiratory distress syndrome into chronic lung disease. European Respiratory Journal 1989; 2 Suppl: 33-7S.
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4. Merrit TA, Cochrane CG, Holcomb K, *et al.* Elastase and alpha-1-proteinase inhibitor activity in tracheal aspirates during respiratory distress syndrome. Role of inflammation in the pathogenesis of bronchopulmonary dysplasia. Journal of Clinical Investigation. 1983; 72: 656-66.
5. Groneck P. Interleukin-8 in pulmonary effluent fluid of preterm infants [letter]. Journal of Pediatrics 1993; 123: 839-40.

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TRACHEAL IL8 AS A PREDICTOR OF CHRONIC LUNG DISEASE

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Hypothesis: Is the neutrophil activating cytokine IL8 associated with chronic inflammation and development of chronic lung disease in the immature infant?
Study type/Setting: Prospective cohort study in a tertiary neonatal service.
Patients: 12 consecutive newborn =< 30 weeks gestation (median 27).
Interventions: 8 infants received surfactant because of respiratory distress syndrome (rescue situation) and all were ventilated using a standard technique.
Measurements: tracheal secretions and washings were collected daily for 7 days at the time of normal endotracheal suction. The IL8 concentrations of the supernatants were measured by a specific RIA (cv 5,13%). Chronic Lung Disease was defined as an oxygen requirement at 28 days of age.
Results: 7 of the 12 infants (58%) developed chronic lung disease typical of bronchopulmonary dysplasia - 2 did not receive surfactant as they initially did not have an oxygen requirement or a diagnosis of RDS. 5 infants (42%) did not develop chronic lung disease - 3 received surfactant because of the initial diagnosis of RDS. All infants survived. The median (range) of IL8 in the tracheal secretions on day 2 in the group who did not develop BPD was 5.7(0.4-20.7)mcg/ml: the corresponding levels in the group going on to develop CLD was 42(22-352) mcg/ml ($p=0.042$, Mann-Whitney U test).
Conclusions: The acute inflammatory cytokine IL8, is present at high concentrations early in the tracheal secretions of babies who go on to develop chronic lung disease.

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Changes in Inflammatory Cells and Inflammatory Mediators in Bronchoalveolar Lavage Fluid after Surfactant

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Background: Tumour Necrosis Factor-α (TNF-α), and interleukin-8 (IL-8) are produced by alveolar macrophages in response to many insults and a high level of IL-8 at 24 hours may predict the development of chronic lung disease.[1] The aim of this study was to explore the inflammatory response to two different surfactants.

Setting: A tertiary neonatal intensive care unit, Edinburgh, Scotland.

Subjects: 23 ventilated newborn infants on the first day of life.

Interventions: Infants with respiratory distress syndrome and an A/a ration < 0.22 were randomised to receive Curosurf (n=12, median gestational age 29 weeks, birthweight 1395g), or Exosurf (n=11, median gestational age 29 week, birthweight 1543g).

Results: Data are medians before and 24 hours after surfactant treatment.

(pre vs post)	**All Infants**	**Exosurf**	**Curosurf**
IL-8 ng/ml	4.4 vs 20.7 (p=0.078)	4.2 vs 23.7 (p=0.04)	6.2 vs 15.6 (p=0.58)
TNF-α pg/ml	0 vs 0 (p=0.29)	0 vs 0 (p=0.29)	0 vs 0 (p=0.29)
% Neutrophils	2 vs 77 (p = 0.001)	22 vs 75 (p=0.06)	1 vs 77.5 (p=0.01)
% Macrophages	0 vs 5 (p = 0.13)	6 vs 5 (p=0.76)	0 vs 8 (p=0.10)

Statistics performed with a Wilcoxon Signed Ranks Test

Conclusions: This preliminary data suggests ther is a larger early inflammatory response of the inflammatory chemokine IL-8 with artificial surfactant.

1. McColm JR and McIntosh N. IL-8 as a predictor of chronic lung disease in premature infants. Pediatric Research 1994 **34:** 28A

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COMPARISON OF TWO TECHNIQUES FOR ASSESSING INFLAMMATORY CELLS IN BRONCHOALVEOLAR LAVAGE FLUID. Jan R McColm, Andrew J Lyon, Linda Middlemist and Neil McIntosh. Department of Child Life and Health, University of Edinburgh, Edinburgh, Scotland, UK.

Background: The development of chronic lung disease is associated with an infiltration of activated immune cells which cause tissue damage by the release of oxygen radicals and enzymes. There has been controversy with most investigators suggesting only the presence of neutrophils contributes to the outcome (1). Most reported studies have used a standard differential stain to distinguish cells based on their morphology, where mature neutrophils are easily recognised but macrophages are not.

Immunohistochemical stains (IHS) rely on cell-specific proteins to distinguish cell types. This should be more accurate and less observer dependent.

Samples: BAL was performed on infants ≤30wks gestation and the sample collected in a mucus trap. The cells were separated by centrifugation, counted and adjusted to $4x10^5$cells/ml. 100µl of sample was added to a cytospin and spun onto slides, which were air dried and either differentially stained or stored at -20°C for later IHS.

Methods: The differential stain was a commercially available kit (Diff Quik, Dade). The IHS used the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique with monoclonal antibodies to the pan-macrophage marker CD68 (Dako) and the neutrophil specific membrane antigens (Sigma). Counts were made on 300 cells per slide and the percentage of cell types present was compared using the Mantel-Haenszel χ^2 test.

Results: Neutrophils counts were not significantly different between techniques (where $n = 23$, $p=0.3$) but there was a significant difference ($n = 38$, $p<0.05$) between macrophage counts, with the IHS counts being higher.

Conclusions: Routine differential staining may not be the best way of assessing macrophage numbers in BAL fluid as morphological differentiation is difficult. A technique based on the identification of cell-specific proteins by IHS is more discriminatory.

1. Arnon S *et al.* Arch Dis Child 1993; 69:44-48.